"Design, Synthesis and anticancer activity of Novel piperidine hydroxamates as HDAC inhibitors"

Dissertation Submitted to

The Tamil Nadu Dr. M.G.R Medical University, Chennai

In partial fulfillment for the requirement of the Degree of

MASTER OF PHARMACY

(Pharmaceutical Chemistry)

April - 2012

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DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

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KOVAI ESTATE, KALAPATTI ROAD,

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Submitted by

R. S. SHANMUGARAJAN

Under the guidance of

Mr. K. Suresh kumar M. Pharm., (Ph.D)

Professor,

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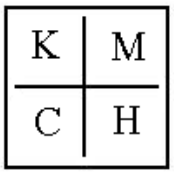

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CERTIFICATE

This is to certify that the dissertation work on **"*Design, Synthesis and anticancer activity of Noval piperidine hydroxamate as HDAC inhibitors."*** submitted by **R.S.Shanmugarajan** is a bonafide work carried out by the candidate under the guidance of **Prof. K. Suresh Kumar, M. Pharm, (Ph.D.,)** to ***The Tamilnadu Dr. M.G.R. Medical University, Chennai,*** in partial fulfillment for the degree of **Master of Pharmacy** in Pharmaceutical Chemistry at the Department of Pharmaceutical Chemistry, KMCH College of Pharmacy, Coimbatore, during the academic year 2011-2012.

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DECLARATION

I do hereby declare that the dissertation work entitled **"*Design, Synthesis and anticancer activity of Noval piperidine hydroxamate as HDAC inhibitors.*"** submitted to ***The Tamilnadu Dr. M.G.R. Medical University, Chennai***, in partial fulfillment for the Degree of **Master of Pharmacy** in Pharmaceutical Chemistry at the Department of Pharmaceutical Chemistry was done by me under the guidance of **Prof. K. Suresh Kumar, M.Pharm., (Ph.D).,** at the Department of Pharmaceutical Chemistry, KMCH College of Pharmacy, Coimbatore, during the academic year 2011-2012.

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EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"*Design, Synthesis and anticancer activity of Noval piperidine hydroxamate as HDAC inhibitors.*"** submitted by **R.S.Shanmugarajan** University Reg.No: **26107138 t**o ***The Tamilnadu Dr. M.G.R. Medical University, Chennai,*** in partial fulfillment for the Degree of Master of Pharmacy in Pharmaceutical Chemistry is a bonafide work carried out by the candidate at the Department of Pharmaceutical Chemistry, KMCH College of Pharmacy, Coimbatore and was evaluated by us during the academic year 2011-2012.

Examination Centre: KMCH College of Pharmacy, Coimbatore.

Date :

Internal Examiner **External Examiner**

Convener of Examinations

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R. S. Shanmugaranjan

ABBREVIATION

HDAC	Histone Deacetylase
Ar	Aromatic
e.g.	Example
%	Percentage
^{1}HNMR	Proton Nuclear Magnetic Resonance
mg	Milligram
ml	Milliliter
µg	Microgram
mm	Millimeter
w/w	Weight by weight
v/v	Volume by volume
µg/ml	Microgram per liter
Hrs	Hours
^{0}C	Degree Celsius
Fig.	Figure
UV-VIS	Ultraviolet and visible spectroscopy
min.	Minutes
IR	Infrared spectroscopy
Std	Standard
TLC	Thin Layer Chromatography
KBr	Potassium bromide
FTIR	Fourier transform infrared spectrometer
IC	Inhibitory concentration
EC	Effective concentration
Cont	Control
DMSO	Dimethyl sulphoxide
VEGF	Vascular endothelial growth factor

EGFR	Epidermal growth factor
Her2	Human Epidermal Growth Factor Receptor 2
$_{P}$53	Cellular tumor antigen
Bcr	B cell receptor
BRCA 1	breast cancer type 1
BRCA 1	breast cancer type 2
HeLa	Cervical cancer cell line
SAHA	Suburanilamide hydroxymic acid
RB	Retinoblastoma
ZBG	Zinc binding group
MTT	micro culture tetrazolinium
PDB	Protein data bank
LW	Lawessons

Abstract

Thirteen compounds were synthesized with piperidine in linker region and hydroxamate as Zinc Binding Group (ZBG). They were screened against (HeLa) human cervical cancer cell-line. Of those, compound 3l (*N*-hydroxy-1-{[(2*E*)-2-(2-hydroxybenzylidene) hydrazinyl] carbonothioyl} piperidine-4-carboxamide) was found a lead compound with promising IC_{50} value of 5.83nM.

Further the lead compound would be evaluated against a panel of cancer cell lines and establish its possible mechanism through enzyme inhibition assay.

1. INTRODUCTION

1.1 CANCER

Cancer is a group of more than 100 diseases characterized by uncontrolled cellular growth as a result of changes in the genetic information of cells. Cells and tissues are complex systems with critical stages and checkpoints to ensure normal growth, development, and function. Normally the division, differentiation, and death of cells are carefully regulated. All cancers start as a single cell that has lost control of its normal growth and replication processes[1].

Human adults are made up of around 10^{13} cells, which are renewed and replaced constantly. About 5–10 per cent of cancers result directly from inheriting genes associated with cancer, but the majority involve alterations or damage accumulated over time to the genetic material within cells. The causes of damage are both endogenous (internal) and exogenous (environmental). Food, nutrition, and physical activity are important environmental factors in the development of cancer[1].

Cancer is characterized by uncontrolled multiplication and spread of abnormal forms of the body's own cells[2]. Cancer is caused in all or almost all instances by mutation or by some other abnormal activation of cellular genes that control cell growth and cell mitosis. The abnormal genes are called oncogenes. As many as 100 different oncogenes have been discovered[3]. It is one of the major causes of the death in developing nations. The term *cancer*, malignant neoplasm (neoplasm means new growth) and malignant tumor are synonymous[2]. The study of tumors is called oncology. Tumors may be cancerous or fatal, or they may be harmless. A cancerous neoplasm is called a malignant tumor or malignancy.

When cells are no longer needed or become a threat to the organism, they undergo a suicidal programmed cell death, or apoptosis. Apoptosis is initiated by activation of a family of proteases called caspases. These are enzymes that are synthesized and stored in the cell as inactive procaspases. The mechanisms of activation of caspases are complex, but once activated; the enzymes cleave and activate other procaspases, triggering a cascade that rapidly breaks down proteins within the cell. The cell thus dismantles itself, and its remains are rapidly digested by neighbouring phagocytic cells. A tremendous amount of apoptosis occurs in tissues that are being re-modelled during development. Even in adult humans, billions of cells die each hour in tissues such as the intestine and bone marrow and are replaced

by new cells. Programmed cell death, however, is precisely balanced with the formation of new cells in healthy adults. Otherwise, the body's tissues would shrink or grow excessively. Recent studies suggest that abnormalities of apoptosis may play a key role in neurodegenerative diseases such as Alzheimer's disease, as well as in cancer and auto-immune disorders. Some drugs that have been used successfully for chemotherapy appear to induce apoptosis in cancer cells[3].

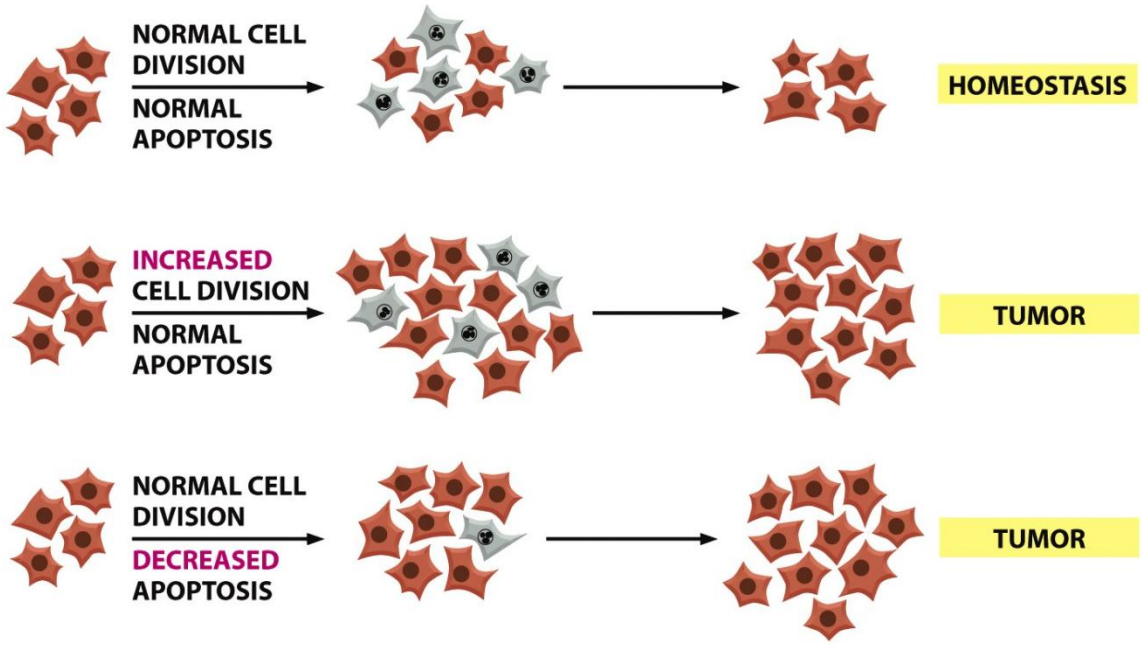

Figure 1: Apoptosis and Tumor

Cancer harms the body when damaged cells divide uncontrollably to form lumps or masses of tissue called tumors (except in the case of leukemia where cancer prohibits normal blood function by abnormal cell division in the blood stream). Tumors can grow and interfere with the digestive, nervous, and circulatory systems and they can release hormones that alter body function. Tumors that stay in one spot and demonstrate limited growth are generally considered to be benign[4].

There are five broad groups that are used to classify cancer[4].

1. Carcinomas are characterized by cells that cover internal and external parts of the body such as lung, breast, and colon cancer.
2. Sarcomas are characterized by cells that are located in bone, cartilage, fat, connective tissue, muscle, and other supportive tissues.

3. Lymphomas are cancers that begin in the lymph nodes and immune system tissues.
4. Leukaemias are cancers that begin in the bone marrow and often accumulate in the bloodstream.
5. Adenomas are cancers that arise in the thyroid, the pituitary gland, the adrenal gland, and other glandular tissues.

1.2 CANCER IN INDIA

In 2007, cancer claimed the lives of about 7.6 million people in the world[4]. Globally the burden of new cancer cases in 2000 was estimated to be around 10 million with more than half of these cases originating from the developing world population. Although estimates vary it is estimated that by the year 2020 there will be almost 20 million new cases. The magnitude of the problem of cancer in the Indian Subcontinent in terms of sheer numbers is the most alarming. From the population census data for India in 1991, 609,000 new cancer cases were estimated to have been diagnosed in the country. This figure had increased to 806,000 by the turn of the century. The estimated age standardized rates per 100,000 were 96.4 for males and 88.2 for females. The most common cancers found in males were cancers of the lung, pharynx, esophagus, tongue and stomach while among females cancers of the cervix, breast, ovary, esophagus and mouth were common Cancer[5].

From the beginning of 2002, 16 oncology clinical trials were granted approval to date. Of these two were phase I studies for chemotherapeutic agents manufactured by Indian Pharmaceutical companies. The studies were for non-small cell lung cancer, head and neck cancer and breast cancer. The year 2003 saw more clinical protocols being submitted for permission to conduct multinational, global studies with India as part of a global drug development plan. Studies that are ongoing include cancers of head & neck, Chronic Myelogenous Leukemia, breast, ovarian, colorectal and lung[5].

Table. 1

Type of cancer	Male (%)	Female (%)	Total (%)
Oral cavity	12.5	13.1	12.8
Pharyngeal cancer	17.6	2.5	9.5
Oesophagus	11.1	6.7	8.7
Larynx	4.6	0.4	2.3
Lung	6.1	1.0	3.3
Urinary bladder	1.4	0.3	0.8
Tobacco cancers	53.3	24.0	37.4

India has become a destination of choice for multinational studies in the field of oncology due to the large patient numbers, improving regulatory processes that are being implemented, investigators who are research and academically inclined and the large number of patients.

1.3 DIFFERENT TYPES OF CANCER[6]

The four most common cancers are:

- Breast Cancer
- Colon Cancer
- Lung Cancer
- Prostate Cancer

Cancers of Blood and Lymphatic Systems:

- Hodgkin's Disease
- Leukemia’s
- Lymphomas
- Multiple Myeloma
- Waldentrom's Disease

Skin cancers:

- Malignant Melanoma
- Skin Cancer

Cancers of Digestive Systems:

- Head and Neck Cancers
- Esophageal Cancer
- Stomach Cancer
- Cancer of Pancreas
- Liver Cancer
- Colon and Rectal Cancer
- Anal cancer

Cancers of Urinary system:

- Kidney Cancer
- Bladder Cancer
- Testis Cancer
- Prostate Cancer

Cancers in women:

- Breast Cancer
- Ovarian Cancer
- Gynecological Cancers
- Chorio carcinoma

Retroperitoneal sarcomas:

- Soft Tissue Tumors
- Thyroid Cancer
- Cancers of Unknown Primary Site

There are more than 100 different types of cancer. Most cancers are named for the organ or type of cell in which they start. Cancers are often referred to by terms that contain a prefix related to the cell type in which the cancer originated and a suffix such as -sarcoma, -carcinoma, or just -oma. Common prefixes include[4]:

- Adeno- = gland
- Chondro- = cartilage
- Erythro- = red blood cell
- Hemangio- = blood vessels
- Hepato- = liver
- Lipo- = fat
- Lympho- = white blood cell
- Melano- = pigment cell
- Myelo- = bone marrow
- Myo- = muscle
- Osteo- = bone
- Uro- = bladder
- Retino- = eye
- Neuro- = brain
-

1.4 CAUSES OF CANCER[1]

A number of different types of exogenous (environmental) factors are known causes of cancer. These include some aspects of food and nutrition that are established as carcinogenic by the International Agency for Research on Cancer:

✓ Endogenous causes

- Inherited germ line mutations
- Oxidative stress
- Inflammation
- Hormones

✓ Exogenous causes

- Tobacco use
- Infectious agents
- Radiation
- Industrial chemicals
- Medication
- Carcinogenic agents in food

1.5 PATHOGENESIS

It is a multifunctional disease, the biology of which is not yet fully understood. Cancer cells manifest, to varying degrees, four characteristics that distinguish them from normal cells. These are uncontrolled proliferation

- Dedifferentiation and loss of function
- Invasiveness
- Metastasis

A normal cell turns into a cancer cell because of mutations in its DNA, which can be acquired or inherited. A good example is breast cancer. Carcinogenesis is a complex multistage process. Increase in the genetic mutations will results eventually in cancer[2].

The activation of proto-oncogenes and inhibition of tumor suppressor genes has been implicated in the pathogenesis of cancer. Apart from this angiogenesis plays an important role in the pathogenesis of cancer and is a common target for most chemopreventive agents.

Angiogenesis is a highly coordinated process regulated by variety of molecules. Vascular endothelial growth factor (VEGF) is the major regulator of tumor angiogenesis in lung adenocarcinoma, responsible for promoting tumor growth and metastasis.

More dangerous, or malignant, tumors form when two things occur[4]:

1. A cancerous cell manages to move throughout the body using the blood or lymph systems, destroying healthy tissue in a process called invasion.
2. That cell manages to divide and grow, making new blood vessels to feed itself in a process called angiogenesis.

When a tumor successfully spreads to other parts of the body and grows, invading and destroying other healthy tissues, it is said to have metastasized. This process itself is called metastasis, and the result is a serious condition that is very difficult to treat.

Tumor Growth and Metastasis[7]

As a tumor grows, nutrients are provided by direct diffusion from the circulation. Local growth is facilitated by enzymes (e.g., proteases) that destroy adjacent tissues. As tumor volume increases, tumor angiogenesis factors are produced to promote formation of the vascular supply required for further tumor growth.

Almost from inception, a tumor may shed cells into the circulation. From animal models, it is estimated that a 1-cm tumor sheds > 1million cells/24 h into the venous circulation. Although most circulating tumor cells die as a result of intravascular trauma, an occasional cell may adhere to the vascular endothelium and penetrate into surrounding tissues, generating independent tumors (metastases) at distant sites. Metastatic tumors grow in much the same manner as primary tumors and may subsequently give rise to other metastases.

Experiments suggest that through random mutation, a subset of cells in the primary tumor may acquire the ability to invade and migrate to distant sites, resulting in metastasis.

Molecular Abnormalities [6&7]

Genes and Cancer

Cells can experience uncontrolled growth if there are damages or mutations to DNA, and therefore, damage to the genes involved in cell division. Four key types of gene are responsible for the cell division process: oncogenes tell cells when to divide, tumor suppressor genes tell cells when not to divide, suicide genes control apoptosis and tell the cell to kill itself if something goes wrong, and DNA-repair genes instruct a cell to repair damaged DNA.

Cancer occurs when a cell's gene mutations make the cell unable to correct DNA damage and unable to commit suicide. Similarly, cancer is a result of mutations that inhibit oncogene and tumor suppressor gene function, leading to uncontrollable cell growth.

Genetic mutations are responsible for the generation of cancer cells. These mutations alter the quantity or function of protein products that regulate cell growth and division and DNA repair. Two major categories of mutated genes are oncogenes and tumor suppressor genes.

Oncogenes:

These are abnormal forms of normal genes (proto-oncogenes) that regulate various aspects of cell growth. Mutation of these genes may result in direct and continuous stimulation of the pathways (e.g., intracellular signal transduction pathways, transcription factors, secreted growth factors) that control cellular growth and division, DNA repair, angiogenesis, and other physiologic processes.

There are > 100 known oncogenes that may contribute to human neoplastic

transformation. For example, the ras gene encodes the Ras protein, which regulates cell division. Mutations may result in the inappropriate activation of the Ras protein, leading to uncontrolled cell growth and division. In fact, the Ras protein is abnormal in about 25% of human cancers. Other oncogenes have been implicated in specific cancers.

These include

- Her2/neu (breast cancer)
- bcr-abl (chronic myelocytic leukemia, B-cell acute lymphocytic leukemia)
- C-myc (Burkett's lymphoma)
- N-myc (small cell lung cancer, neuroblastoma)

Specific oncogenes may have important implications for diagnosis, therapy, and prognosis (see individual discussions under the specific cancer type).

Oncogenes typically result from acquired somatic cell mutations secondary to point mutations (eg, from chemical carcinogens), gene amplification (eg, an increase in the number of copies of a normal gene), or translocations.

Tumor suppressor genes:

Genes such as the p53 gene play a role in normal cell division and DNA repair and are critical for detecting inappropriate growth signals in cells. If these genes, as a result of inherited or acquired mutations, become unable to function, genetic mutations in other genes can precede unchecked, leading to neoplastic transformation.

p53, important regulatory protein, prevents replication of damaged DNA in normal cells and promotes cell death (apoptosis) in cells with abnormal DNA. Inactive or altered p53 allows cells with abnormal DNA to survive and divide. Mutations are passed to daughter cells, conferring a high probability of neoplastic transformation. The p53 gene is defective in many human cancers. As with oncogenes, mutation of tumor suppressor genes such as p53 or RB (retinoblastoma) in germ cell lines may result in vertical transmission and a higher incidence of cancer in offspring.

Examples of these genes are:

RB gene; if this genes goes bad, it can lead to the development of Retinoblastoma, Bone, Breast, Lung, Prostate, Bladder and other cancers.

p53 gene; p53 suppresser gene can arrest replication of cells with damaged genes until normal repair process has taken place. If cells with damaged genes grow and replicate, they may result in a cancer. *p53 gene* suppresses the growth of such cells. If this gene goes mutation, it can lead to the development of Breast, Colon, Leukemia, soft tissue sarcomas, and many other cancers.

BRCA1; located in chromosome 17, if this genes goes bad, it can be associated with a very high risk of developing Breast cancer.

BRCA2; located in chromosome 13, if this genes goes bad, it can be associated with a very high risk of developing Breast cancer.

Cell Cycle in Cancer

The cell cycle, the process by which cells progress and divide, lies at the heart of cancer. In normal cells, the cell cycle is controlled by a complex series of signaling pathways by which a cell grows, replicates its DNA and divides. This process also includes mechanisms to ensure errors are corrected, and if not, the cells commit suicide (apoptosis). In cancer, as a result of genetic mutations, this regulatory process malfunctions, resulting in uncontrolled cell proliferation[8].

The cell cycle involves a complex series of molecular and biochemical signalling pathways. As illustrated in the Fig. 2, the cell cycle has four phases:

Cell cycle is an ordered series of events consisting of several sequential phases: G_1, S, G_2 and M[2].

- M is the phase of mitosis
- S is the phase of DNA synthesis
- G_1 is the gap between the mitosis that gave rise to the cell and the S phase; during G_1, the cell is preparing for DNA synthesis
- G_2 is the gap between S phase and the mitosis that will give rise to two daughter cells; during G_2, the cell is preparing for the mitotic division into two daughter cells.

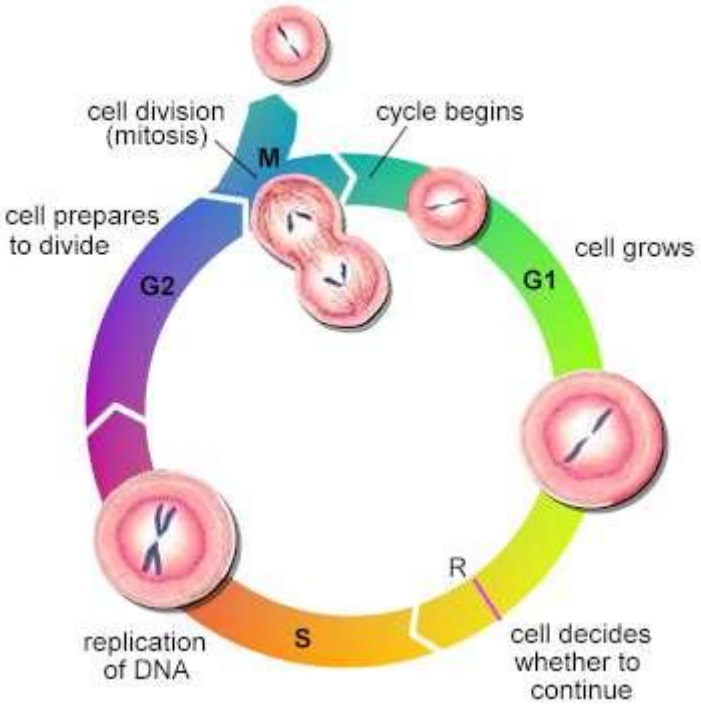

Figure.2 Normal cell cycle

As a cell approaches the end of the G1 phase it is controlled at a vital checkpoint, called G1/S, where the cell determines whether or not to replicate its DNA. At this checkpoint the cell is checked for DNA damage to ensure that it has all the necessary cellular machinery to allow for successful cell division. As a result of this check, which involves the interactions of various proteins, a ‘‘molecular switch’’ is toggled on or off. Cells with intact DNA continue to S phase; cells with damaged DNA that cannot be repaired are arrested and ‘‘commit suicide’’ through apoptosis, or programmed cell death. A second such checkpoint occurs at the G2 phase following the synthesis of DNA in S phase but before cell division in M phase. Cells use a complex set of enzymes called kinases to control various steps in the cell cycle. Cyclin Dependent Kinases, or CDKs, are a specific enzyme family that use signals to switch on cell cycle mechanisms. CDKs themselves are activated by forming complexes with cyclins, another group of regulatory proteins only present for short periods in the cell cycle. When functioning properly, cell cycle regulatory proteins, Including CDKs and cyclins, act as the body’s own tumor suppressors by inducing the death of damaged cells. Genetic mutations causing the malfunction or absence of one or more of the regulatory proteins at cell cycle checkpoints can result in the ‘‘molecular switch’’ being turned permanently on, permitting uncontrolled

multiplication of the cell, leading to carcinogenesis, or tumor development[8]. p53 is a protein that functions to block the cell cycle if the DNA is damaged. If the damage is severe this protein can cause apoptosis[9].

- p53 levels are increased in damaged cells. This allows time to repair DNA by blocking the cell cycle.
- A p53 mutation is the most frequent mutation leading to cancer. An extreme case of this is Li Fraumeni syndrome, where a genetic a defect in p53 leads to a high frequency of cancer in affected individuals.

1.6 CANCER DRUG DISCOVERY

Traditionally, cancer drugs were discovered through large-scale testing of synthetic chemicals and natural products against rapidly proliferating animal tumor systems, primarily murine leukemias. Most of the agents discovered in the first two decades of cancer chemotherapy (1950 to 1970) interacted with DNA or its precursors, inhibiting the synthesis of new genetic material or causing irreparable damage to DNA itself. In recent years, the discovery of new agents has extended from the more conventional natural products of which target the proliferative process, to entirely new fields of investigation. These new fields represent the harvest of new knowledge about cancer biology, leading to the discovery of drugs that inhibit novel molecular targets[10].

Similarly targeted immunological approaches use monoclonal antibodies against tumor-associated antigens such as *her-2/neu* receptor in breast cancer cells, often in conjunction with cytotoxic drugs. These examples emphasize that both the strategy for drug evaluation and the routine care of cancer patients are likely to undergo revolutionary changes as entirely new treatment approaches arise from new knowledge of cancer biology[10].

Cancer therapies targeted developing cell cycle-based mechanism that emulates the body's natural process in order to stop the growth of cancer cells. This approach can limit the damage to normal cells and the accompanying side effects caused by conventional chemotherapeutic agents[8].

1.7 TARGETS OF CANCER RESEARCH[2]:

Proteins

- Nucleic acids and their precursors
- Tublin (Micro tubular protein)

Enzymes

- DNA topoisomerase –I &II
- 5-α Reductase,
- DNA polymerase
- Ribonucleoside diphosphate reductase
- Histone deacetylase (HDAC)
- Thiomidilate synthatase,
- Thimydilate synthase.

Hormones

- Estrogens,
- Testosterone
- Androgen,
- Progestin,

Genes

- p53,
- EGFR
- VEGF
- Onco gene

1.8 HISTONE DEACETYLASE (HDAC) ENZYME [10&11]

Introduction

The balance of histone acetylation and deacetylation is an epigenetic layer with a critical role in the regulation of gene expression. Histone acetylation induced by histone acetyltransferases (HATs) is associated with gene transcription, while

histone hypoacetylation induced by histone deacetylase (HDAC) activity is associated with gene silencing. Altered expression and mutations of genes that encode HDACs have been linked to tumor development

Since them both induce the aberrant transcription of key genes regulating important cellular functions such as cell proliferation, cell-cycle regulation and apoptosis. Thus, HDACs are among the most promising therapeutic targets for cancer treatment, and they have inspired researchers to study and develop HDAC inhibitors.

Biological function of (HDAC)

HDACs are key enzymes regulating important cell processes such as cell-cycle progression and apoptosis. Another way by which HDACs are recruited to DNA independently of DNA methylation involves the interaction with transcription factors and nuclear receptors. Focusing on the interaction with transcription factors, HDAC1 andHDAC2 are involved in transcriptional repression regulated by the retinoblastoma protein Rb E2Fis a family of transcription factors involved in cell-cycle control.E2F-containing promoters are repressed by members of the Rb family that are recruited by a physical interaction with the E2F protein. One possibility is that the repression of E2f-regulated promoters by Rb implies the recruitment of HDACs to the E2F-containing promoters Treatment with TSA, a classical HDAC inhibitor, prevents the Rb-mediated repression of gene transcription

Role of HDACs in cancer

A typical characteristic of human cancer is the deregulation of DNA methylation and post translational histone modifications, in particular histone acetylation, which has the fatal consequence of gene transcription deregulation. The role of HDACs in cancer is not restricted to their contribution to histone deacetylation, but also to their role in deacetylation of non-histone proteins. For example, HDAC1interacts with the tumor suppressor p53 and deacetylates it in vivo and in vitro p53 is phosphorylated and acetylated under stress conditions. Since lysine residues acetylated in p53 overlap with those that are ubiquitinated, p53 acetylation serves to promote protein stability and activation, inducing checkpoints in the cell-division cycle, permanent cell-division arrest, and cell death.

Mutations or alterations that induce loss of function of class I HDACs may contribute to cancer development. The tumor-suppressor gene RB requires the recruitment of class I HDACs to repress gene transcription Thus, the loss of class I

HDAC activity could induce the expression of genes regulated by Rb, thereby suppressing their protective role in tumor development

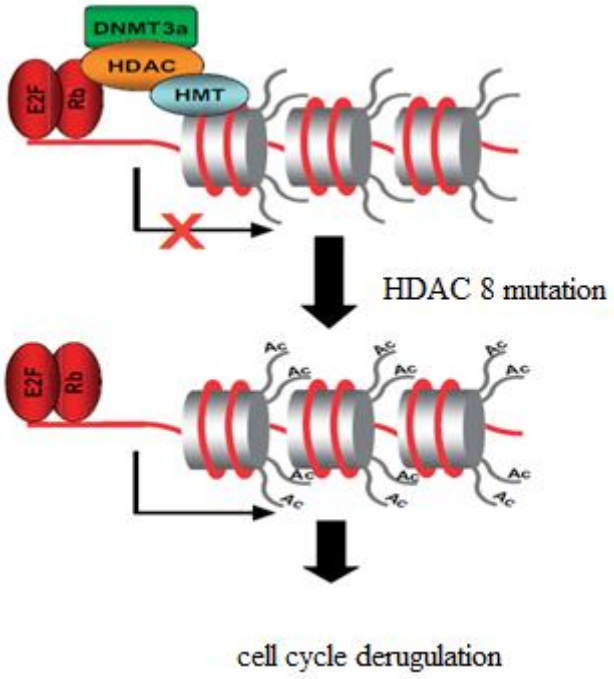

Figure.3

A model showing a possible effect of HDAC2 mutation in cancer development. Class I HDACs are involved in gene transcription-repression mediated by retinoblastoma protein. The lost of HDAC2 function could induce the hyper acetylation and re expression of genes regulated by retinoblastoma protein Rb, and with crucial functions in cell cycle regulation.

Table.2

Classification of (HDAC) enzyme[13]

GROUP	SIZE	LOCATION CHROMOSOME	CELLULAR DISTRIBUTION	COMPLEX	ROLE
			Class I (type Rpd3)		
HDAC 1	483	Ip34	N	Sin3, NURD	TC
HDAC 2	488	6q21	N	Sin3, NURD	TC
HDAC 3	423	5q31	N	NCOR1/NCOR2-GPS2-TBL1X	TC
HDAC8	377	Xp13	N		TC
			CLASS IV		
HDAC11	347	3p25.2	N		
			CLASS II (TYPE had 1)		
HDAC4	1084	q37.2	N,C	NCOR1/NCOR2	TC
HDAC5	1122	17q21	N,C		TC
HDAC6	1215	xp11.22	N,C		
HDAC7	855	12q13	N,C	Sin3, NCOR2	TC
HDAC9	1011	p21-p15	N,C		
HDAC10	669	22q13.31	N,C	NCOR2	TC

Size is expressed in amino acid number, N: nuclear, C: cytoplasm, TC: Transcription corepressor.

1.9 MOLECULAR MODELLING (14)

- Computational chemistry/molecular modeling is the science (or art) of representing molecular structures numerically and simulating their behavior with the equations of quantum and classical physics. Computational chemistry programs allow scientists to generate and present molecular data including geometries (bond lengths, bond angles and torsion angles), energies (heat of formation, activation energy, etc.), electronic properties (moments, charges and ionization potential and electron affinity), spectroscopic properties (vibrational modes, chemical shifts) and bulk properties (volumes, surface areas, diffusion, viscosity, etc.). As with all models however, the chemist's

intuition and training is necessary to interpret the results appropriately. Comparison to experimental data, where available, is also important to guide both laboratory and computational work.

- **Molecular Graphics:** It allows the 3D visualization and manipulation of structure to allow visualization of different parts of molecule, to change the orientation of specific function while holding other constant and to look at other different feasible conformations. Stereochemistry relationship including detailed measurement of molecular geometry and conformations, calculations of electron densities, electrostatic potentials, energies and direct comparison of the key structural features of a range of biologically active structures can be done by molecular graphics.
- **Computational Chemistry:** It is concerned with the simulation of atomic and molecular properties of compounds of medicinal interest through equations and with the numeric methods used to solve these equations on the computer.
- **Statistical Modelling:** It encompasses the search for quantitative relationship between the structure or properties of a series of compound and their resultant biological activities**.**

Functions of Molecular Modelling

1. Structure Generation: Molecular structure may be generated by a variety of procedures:

- The crystal structure (if available) can be loaded from Cambridge crystallographic data file.
- 2D structure can be converted to 3D by software programs such as Chem Office
- The structure can be built up by stitching together small fragments.
- By modifying a known structure.

2. Structure Visualization: One of the most popular uses of molecular modelling system is to visualize molecular structures in a desired form. Different methods are here to represent molecular structures:

- Ball and Stick representation
- Colored Stick representation
- Space Fill representation
- Stereo Line representation

3. Conformation Generation: The biological activity of a drug molecule is supposed to depend on one single, unique conformation hidden among all the low energy conformations. Only the bioactive conformation can bind to the specific macromolecular environment at the active site of the receptor protein. It is widely accepted that bioactive conformation is not necessarily identical with the lowest energy-conformation. However, on the other hand it cannot be the conformation that is so high in energy that it is excluded from the population of conformations in solution. With the help of molecular modelling various conformations of a molecule can be explored. There are three methods for exploring of the conformations of a molecule:

- Systematic or Grid search.
- Model building methods.
- Random methods

Molecular Interaction[16] **(Docking)**

The interaction of a drug with its receptor is a complex process. Many factors are involved in the intermolecular association such as hydrophobic; Van der Waal's, hydrogen bonding and electrostatic forces.

The process of "DOCKING" a ligand to binding sites tries to mimic the natural course of interaction of the ligand and its receptor via a lowest energy pathway. Usually the receptor is kept rigid while the conformation of the drug molecule is allowed to change. The molecules are physically moved closer to one another and the preferred docked conformation is minimized. Molecular docking is a study of how two or more molecular structures, for example drug and enzyme or receptor of protein, fit together. The most important application of docking software is virtual screening. In virtual screening the most interesting and promising molecules are selected from an existing database for further research. This places demands on the used computational method; it must be fast and reliable.

Glide (Schrödinger) [18]

Glide uses a hierarchical series of filters to search for possible locations of the ligand in the active site region of the receptor. The shape and properties of the receptor are represented by a grid using several different sets of fields that provide progressively more accurate scoring of the ligand poses. Conformational flexibility is handled in Glide by an extensive conformational search, augmented by a heuristic screen that rapidly eliminates unsuitable conformations, such as

conformations that have long range internal hydrogen bonds and high energy conformers. Glide can also dock sets of pre-computed conformations. However, Glide offers its greatest value when conformations are generated internally. For each core conformation (or for rigid docking, each ligand), an exhaustive search of possible locations and orientations is performed over the active site of the protein. The search begins with the selection of "site points" on an equally spaced 2 Å grid that covers the active site region.

The second stage of the hierarchy begins by examining the placement of atoms that lie within a specified distance of the line drawn between the most widely separated atoms (the *ligand diameter*). This is done for a pre-specified selection of possible orientations of the ligand diameter (Step 2a). If there are too many steric clashes with the receptor, the orientation is skipped. Next (Step 2b), rotation about the ligand diameter is considered, and the interactions of a subset consisting of all atoms capable of making hydrogen bonds or ligand-metal interactions with the receptor are scored (*subset test*). If the score is good enough, all interactions with the receptor are scored (Step 2c). The scoring in these three tests is carried out using Schrödinger's discretized version of the ChemScore empirical scoring function. Much as for ChemScore itself, this algorithm recognizes favourable hydrophobic, hydrogen-bonding, and metal-ligation interactions, and penalizes steric clashes. This stage is called "greedy scoring," because the actual score for each atom depends not only on its position relative to the receptor but also on the best possible score it could get by moving ±1 Å in *x*, *y*, or *z*. This is done to mute the sting of the large 2 Å jumps in the site-point/ligand-centre positions. The final step in Stage 2 is to re-score the top greedy scoring poses via a "refinement" procedure (Step 2d), in which the ligand as a whole is allowed to move rigidly by ±1 Å in the Cartesian directions.

Only a small number of the best refined poses (typically 100-400) is passed on to the third stage in the hierarchy—energy minimization on the pre-computed OPLS-AA van der Waals and electrostatic grids for the receptor. The energy minimization typically begins on a set of van der Waals and electrostatic grids that have been "smoothed" to reduce the large energy and gradient terms that result from too-close interatomic contacts. It finishes on the full-scale OPLS-AA non-bonded energy surface ("annealing"). This energy minimization consists only of rigid-body translations and rotations when external conformations are docked. When conformations are generated internally, however, the optimization also includes

torsional motion about the core and end-group rotatable bonds. Unless otherwise specified, a small number of the top-ranked poses are then subjected to a sampling procedure in which alternative local minima core and rotamer-group torsion angles are examined to try to improve the energy score.

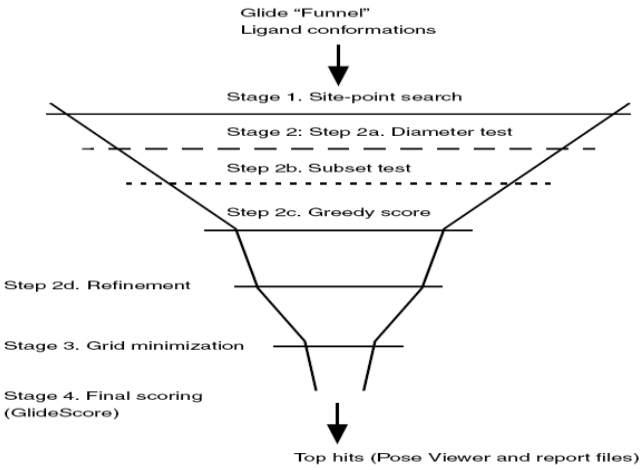

Figure.4 Glide docking hierarchy

Energy Calculation and Energy Minimization [17]

It was hypothesized that a ligand or drug binds to the enzyme or receptor in its most stable form i.e. 'minimum energy state' form and hence properties of this energy optimized molecule will give the information regarding physicochemical requirements which govern the biological activities. This forms the basis of energy calculation and energy minimization. Energy minimizing procedures can be divided in to two classes:

- First derivative techniques (e.g. Steepest Descent, Conjugate Gradient, and Powell method).
- Second derivative techniques (e.g. Newton-Raphson)

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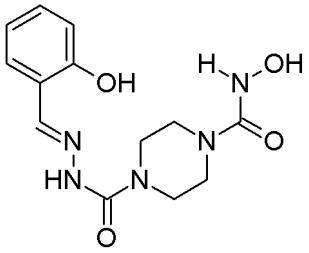
2. LITERATURE REVIEW

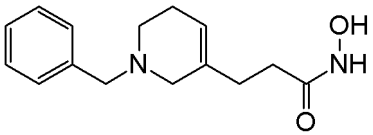
Anti-cancer

Chetan[1] *et al* (2010) had synthesised and reported piperezine hydroxymic acid derivatives as HDAC8 inhibitors. They screened for their anticancer activity against HL60 human promyelocytic leukaemia cell line due to the presence of pharmacophoric features Compound 1 had IC_{50} of 0.6l µM.



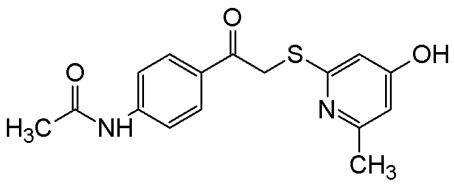
(1)

Kim[2] *et al* (2006) had reported the δ-Lactam-based hydroxamic acid, inhibitors of histone deacetylase (HDAC). The compound (2) exhibited growth inhibitory activity on five human tumor cell lines, showing good sensitivity on the MDA-MB-231 breast tumor cell line.



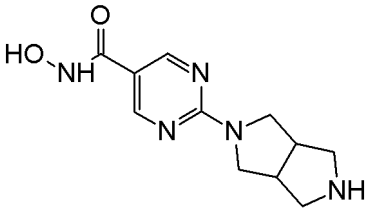
(2)

Kemp[3] *et al* (2011) they are investigated a small molecule with a novel hydroxy-pyrimidine scaffold that inhibits multiple HDAC enzymes and modulates acetylation levels in cells. Compound (3) has found to be potent in order to evaluate structure–activity relationships.



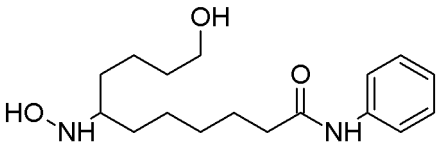
(3)

Donald[4] *et al* (2010) has synthesized and reported the novel HDAC series demonstrating inhibitory activity in cell proliferation assays is described. The compound (4) was good activity against human colon cancer cell line.



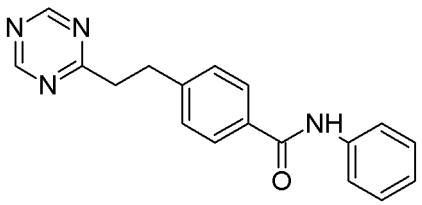
(4)

Bieliauskas[5] *et al* (2007) had synthesised and reported a small molecule library with a variety of substituent's attached adjacent to the metal binding hydroxamic acid of SAHA. The compound (5) has more inhibitory activities in the nanomolar range.



(5)

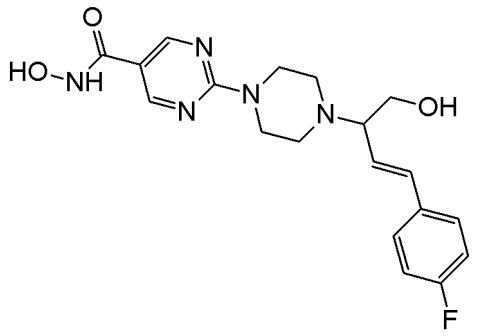
Paquin[6] *et al* (2008) had synthesed and biological evaluated of a variety of 4-(heteroarylaminomethyl)-N-(2-aminophenyl)-benzamides is presented herein. The compound (6) showed the best HDAC1 enzyme inhibitory action and *in vitro* anti-proliferative activities with IC_{50} values below micro molar range.



(6)

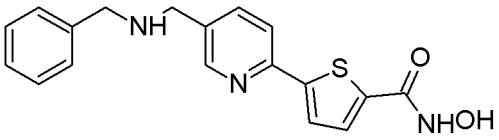
Angibaud[7] *et al* (2009) had designed and synthesised and reported designing 5-pyrimidyl hydroxamic acid anti-cancer agents; they have identified a new series of potent histone deacetylase (HDAC) inhibitors. The compound (7) exhibit enzymatic

HDAC inhibiting properties with IC_{50} values in the nanomolar range and inhibit tumor cell proliferation at similar levels.



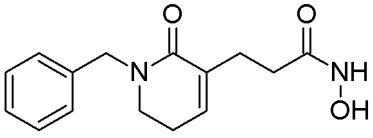
(7)

Price[8] *et al* (2007) had synthesed a series of thienyl-based hydroxamic acids that included ADS100380 and ADS102550 led to the identification of the 5-pyridin-2-yl-thiophene-2-hydroxamic acid. Substitution at the 5- and 6-positions of the pyridyl ring of compound (8) has excellent enzyme inhibition and anti proliferative activity.



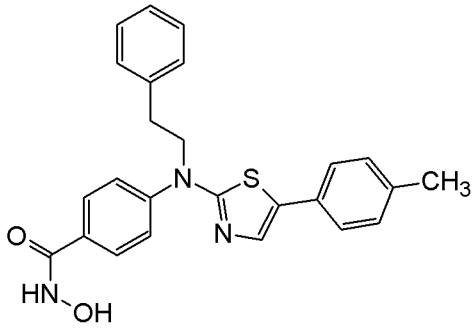
(8)

Choi[9] *et al* (2011) had synthesized δ -lactam core HDAC inhibitors which showed potent HDAC inhibitory activities as well as cancer cell growth inhibitory activities. Hydrophobic and bulky cap groups increase potency of HDAC inhibition because of hydrophobic interaction between HDAC and inhibitors. In overall, the compound (9) γ -lactam based HDAC inhibitors showed more potent than δ-lactam analogue.



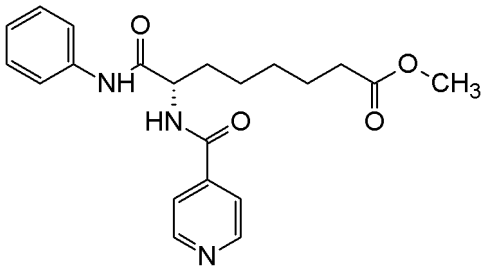
(9)

Hong Su[10] *et al* (2009) had reported some novel N-hydroxybenzamide-based HDAC inhibitors. Introducing branched hydrophobic groups at the capping group, and their inhibition activity against HDACs and anti-proliferation activity in four tumor cell lines were determined. Compound (10) was more potent in human HDAC1 and HDAC4 to evaluate their selectivity profile.



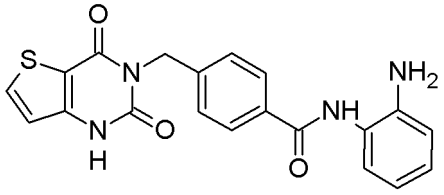
(10)

Belvedere[11] *et al* (2007) had synthesised and reported the HDAC inhibitory activity of a vorinostat-derived series of substrate-based HDAC inhibitors (2-L-aminosuberic acid) from hydroxymic acid. The (11) compound was posses the optimal activity compare with vorinistate.



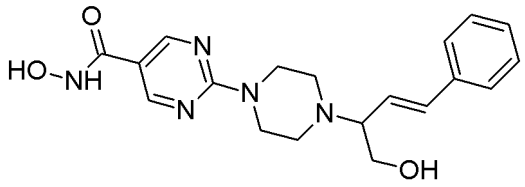
(11)

Vaisburg[12] *et al* (2007) has synthesised and reported the variety of compounds of N-(2-amino-phenyl)-4-(heteroarylmethyl)-benzamides. Compound (12) was found activity against HDAC1 and showed *in vivo* activity in various human tumor xenograft models in mice.



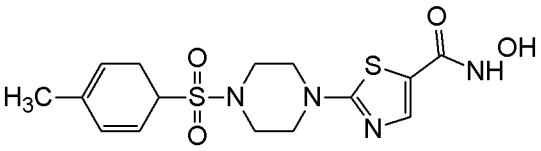
(12)

Angibaud[13] *et al* (2010) have been synthesised and reported 5-pyrimidylhydroxamic acid anti-cancer agents, they have identified a new series of potent histone deacetylase (HDAC) inhibitors. The compound (13) exhibited enzymatic HDAC inhibiting properties with IC_{50} values in the nanomolar range.



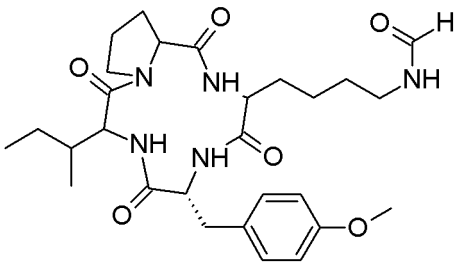
(13)

Anandan[14] *et al* (2007) had reported a series of hydroxamic acid-based histone deacetylase (HDAC) inhibitors characterized by a zinc chelating head group attached directly to a thiazole ring. Compound (14) is potently inhibiting an HDAC enzyme and antiproliferative activity against the breast cancer cell line MCF7.



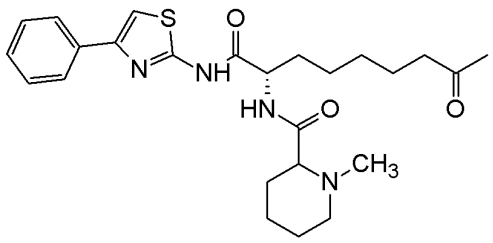
(14)

Nishino[15] *et al* (2004) had synthesised and reported cyclic tetra peptide retrohydroxamic acids as histone deacetylase (HDAC) inhibitors and evaluated the inhibitory activity. The compound (15) has more potential as anticancer drugs.



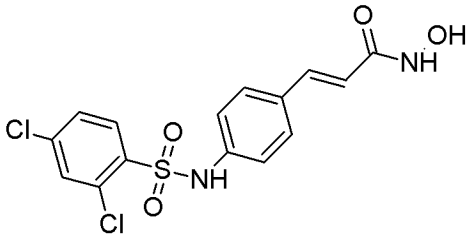
(15)

Jones[16] *et al* (2006) had synthesised and reported the L-2-amino-8-oxodecanoic acid (L-Aoda) derivatives and identified a small acyclic lead molecule with the unusual ketone zinc binding group. The compound (16) was more potent against HDAC enzyme.



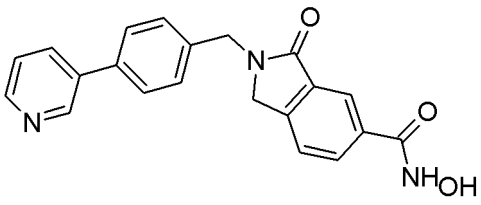
(16)

Lavoie[17] *et al* (2001) had synthesised and reported a series of sulfonamide hydroxamic acid derivatives. Further optimization of this series by substitution of the terminal aromatic ring yielded the compound (17) was showed good *in vitro* and *in vivo* activities.



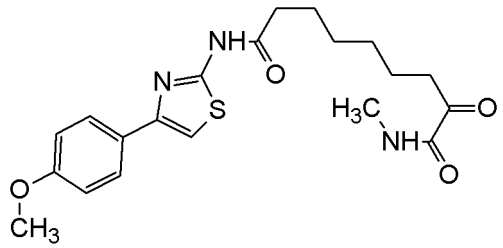
(17)

Lee[18] *et al* (2007) had synthesised and reported the hydroxamic acid derivatives bearing a 4-(3-pyridyl) phenyl group as a cap structure. A representative compound (18) showed more potent growth-inhibitory activity against pancreatic cancer cells and greater upregulation of p21WAF1/CIP1 expression than the clinically used HDAC inhibitor suberoylanilide hydroxamic acid.



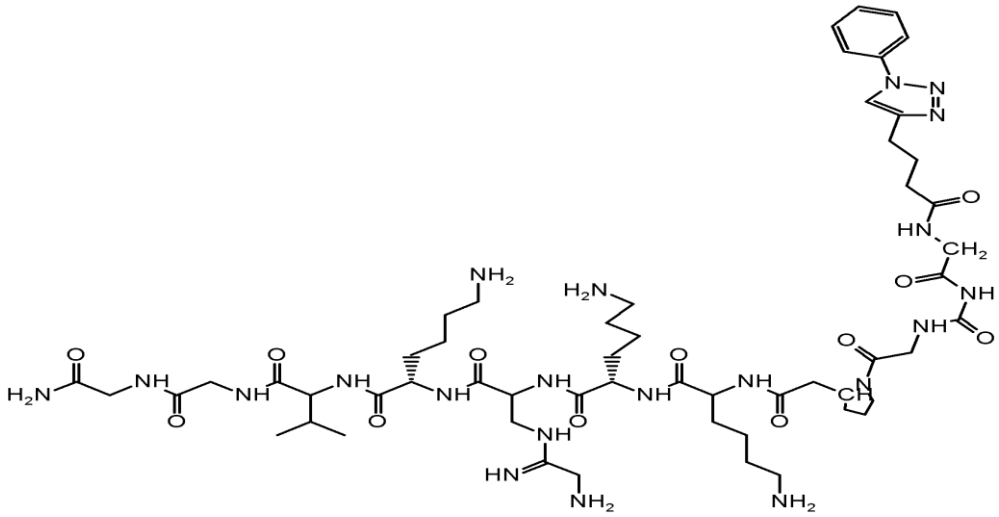
(18)

Wada[20] *et al* (2003) has synthesised Keto ester and amides of thiazole as histone deacetylase inhibitor. Nanomolar inhibitors against the isolated enzyme and sub-micromolar inhibitors of cellular proliferation were obtained. The keto amide (20) also exhibited significant anti-tumor effects in an in vivo tumor model.



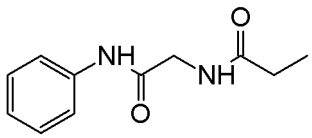
(19)

Canzoneri[19] *et* al (2009) had synthesised and reported the new class of histone deacetylase (HDAC) inhibitors derived from conjugation of a suberoylanilide hydroxamic acid-like aliphatic-hydroxamate pharmacophore to a nuclear localization signal peptide. Compound (19) showed more potent activity against HDAC6 and HDAC 8 when compared to SAHA.



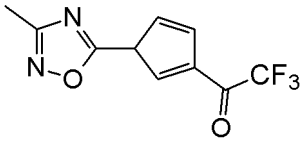
(20)

Suzuki[21] *et al* (2005) had designed and synthesised several suberoylanilide hydroxamic acid (SAHA)-based compounds the catalytic mechanism of HDACs.compound (21) was found to be potent as SAHA.



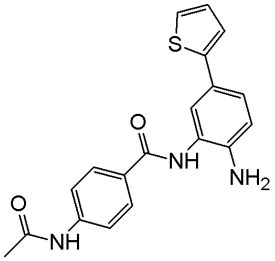
(21)

Scarpelli[22] *et al* (2001) had synthesised and reported the novel series of 5-(trifluoroacetyl) thiophene-2-carboxamides as potent and selective class II HDAC inhibitors. The compound (22) is valid lead compound.



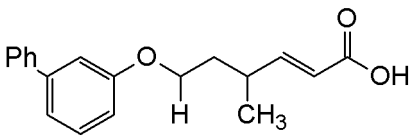
(22)

Methot[23] *et al* (2008) had synthesised and reported the the initial exploration of novel selective HDAC1/HDAC2 inhibitors (SHI-1:2). Structures 23 was exhibit enhanced intrinsic activity against HDAC1 and HDAC2.the compound (23) is more active against (HDAC2) enzyme.



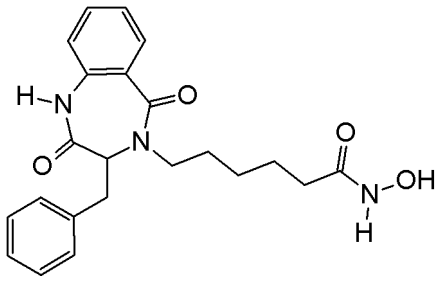
(23)

Pabba[24] *et al* (2011) had synthesised and reported the series of (HDAC) inhibitors with aryl ether and aryl sulfone residues at the terminus of a substituted, unsaturated 5-carbon spacer moiety evaluated. Compound 24 is more potent HDAC inhibitors with activities at low nanomolar levels.



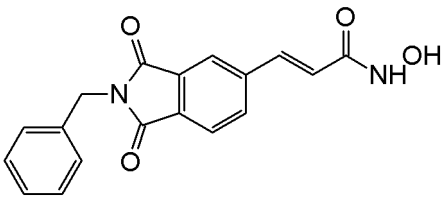
(24)

Loudni[25] *et al* (2007) had synthesised substituted 1, 4-benzodiazepine-2, 5-dione moieties as cyclic peptidemimic cap structures, and a hydroxamate side chain. The compound (25) exhibited promising HDAC-inhibitory activities



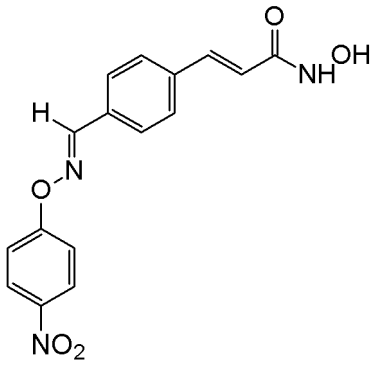
(25)

Shinji[26] *et al* (2006) had synthesised and reported a series of hydroxamic acid derivatives bearing a cyclic amide/imide group as a linker and/or cap structure. Compound (26) showed class-selective potent histone deacetylase (HDAC)-inhibitory activity.



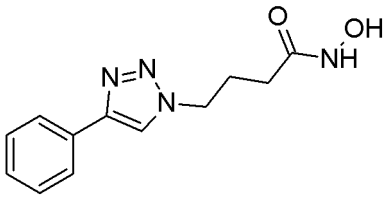
(26)

Giannini[27] *et al* (2009*)* had synthesised the N-hydroxy-(4-oxime)-cinnamide scaffold, and screened against cancer cell line NB4, H460 & HCT116. Compound (27) was more potent against that three cancer cell lines.



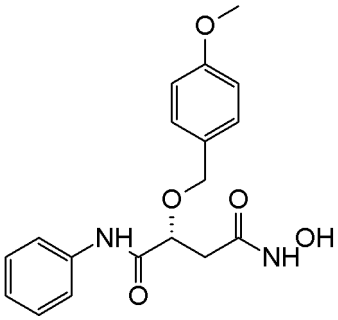
(27)

Chen[28] *et al* (2008) had synthesised 1, 2, 3- triazole ring as a surface recognition cap group linking moiety in suberoylanilide hydroxamic acid-like (SAHA-like) HDAC inhibitors. The compound (28) is more potent against HDAC inhibition and cell growth inhibitory activities.



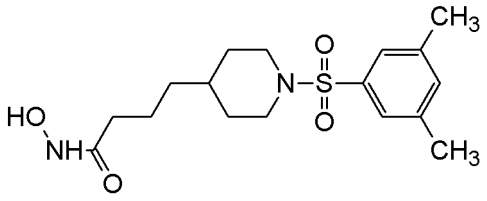
(28)

Hanessian[29] *et al* (2007) explored a series of alkoxy ethers with variation of the length of the aliphatic chain of suberoylanilide hydroxamic acid (SAHA, vorinostat). The compound (29) showed the best activity against human cancer cell line NB4, H460 & HCT-116.



(29)

Rossi[30] *et al* (2011) had reported the N-substituted 4-alkyl piperidine hydroxamic acid. The compound (30) was identified as more potent against cancer cell line HCT-116.



(30)

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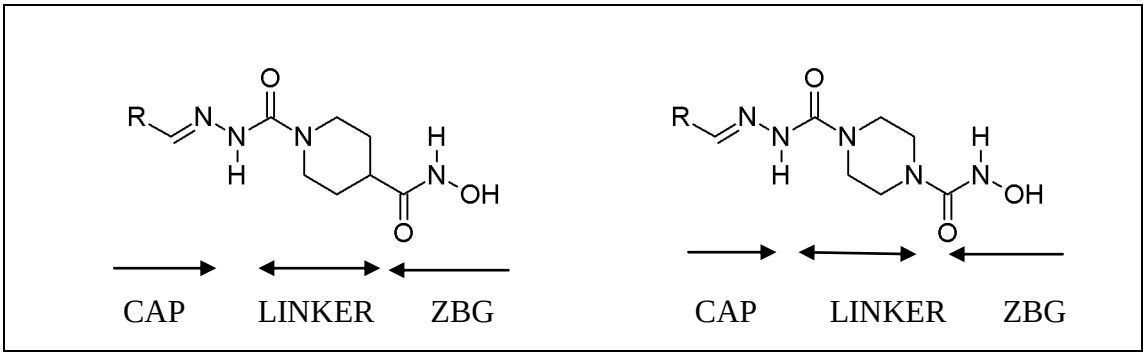
3. METHADOLOGY

3. 1. RESEARCH ENVISAGED

Histone deacetylases (HDACs) are enzymes involved in many important biological functions. They have been linked to a variety of cancers, psychiatric disorders. Histone deacytylase is the important target for cancer research. HDAC has majorly two types. Sub type (HDAC) 8 is more efficient target for hydroxymate derivatives. Literatures revealed that various aryl and hetroaryl, derivatives are an ideal linker to design HDAC inhibition. Recently chetan[1] et al explored piperazine as a linker for hydroxamic acid derivative as HDAC inhibitors with IC50 of micromolar range. This prompted us to synthesize a newer analogue of piperazine isoster piperidine as a newer hydraxamic acid derivatives with enhanced spectrum of activity which were designed by molecular modeling software, based on this the best molecule would be synthesized and evaluated for the Histone deacetylases (HDACs) inhibitory action



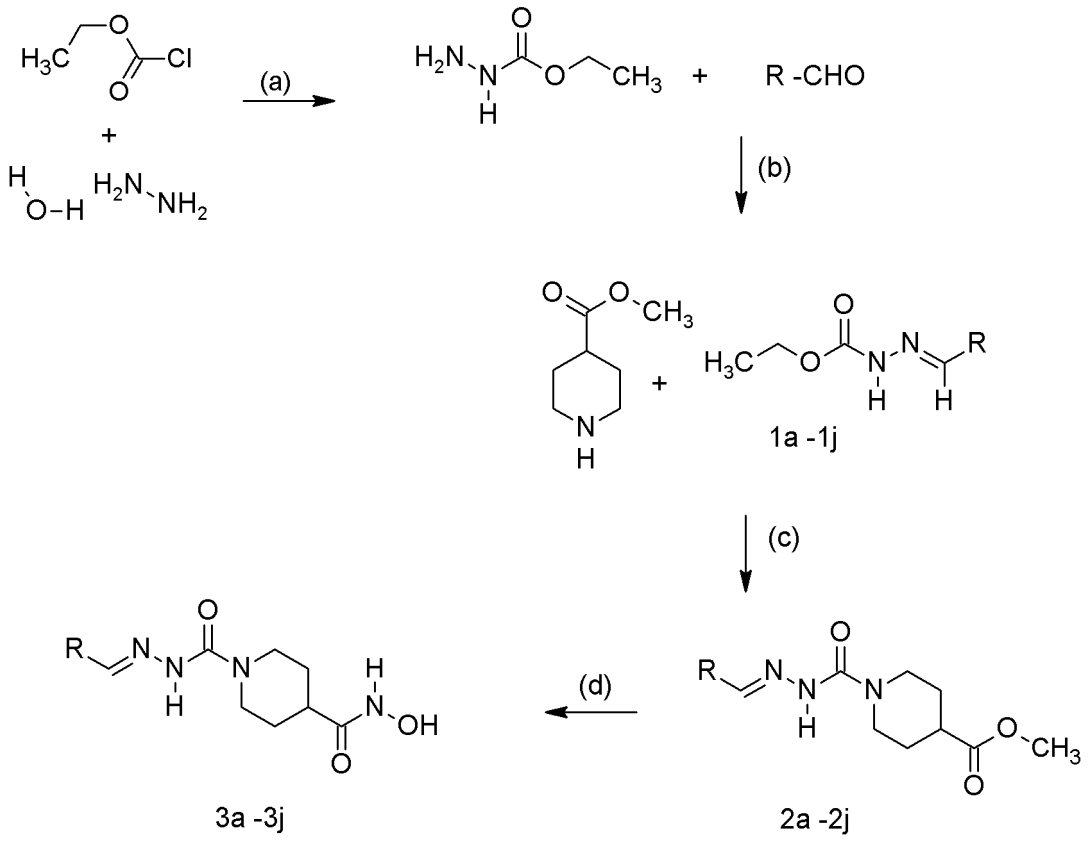
From above consideration we

- Synthesis of some novel piperidine hydroxamate derivatives.
- Characterization of synthesized compounds by various analytical techniques like TLC, FTIR, H^1NMR and Mass Spectral studies.

BIOLOGICAL SCREENING

- Screening for *in vitro* anticancer activity against (HeLa) cervical cancer cell line by MTT assay method.

Scheme 1

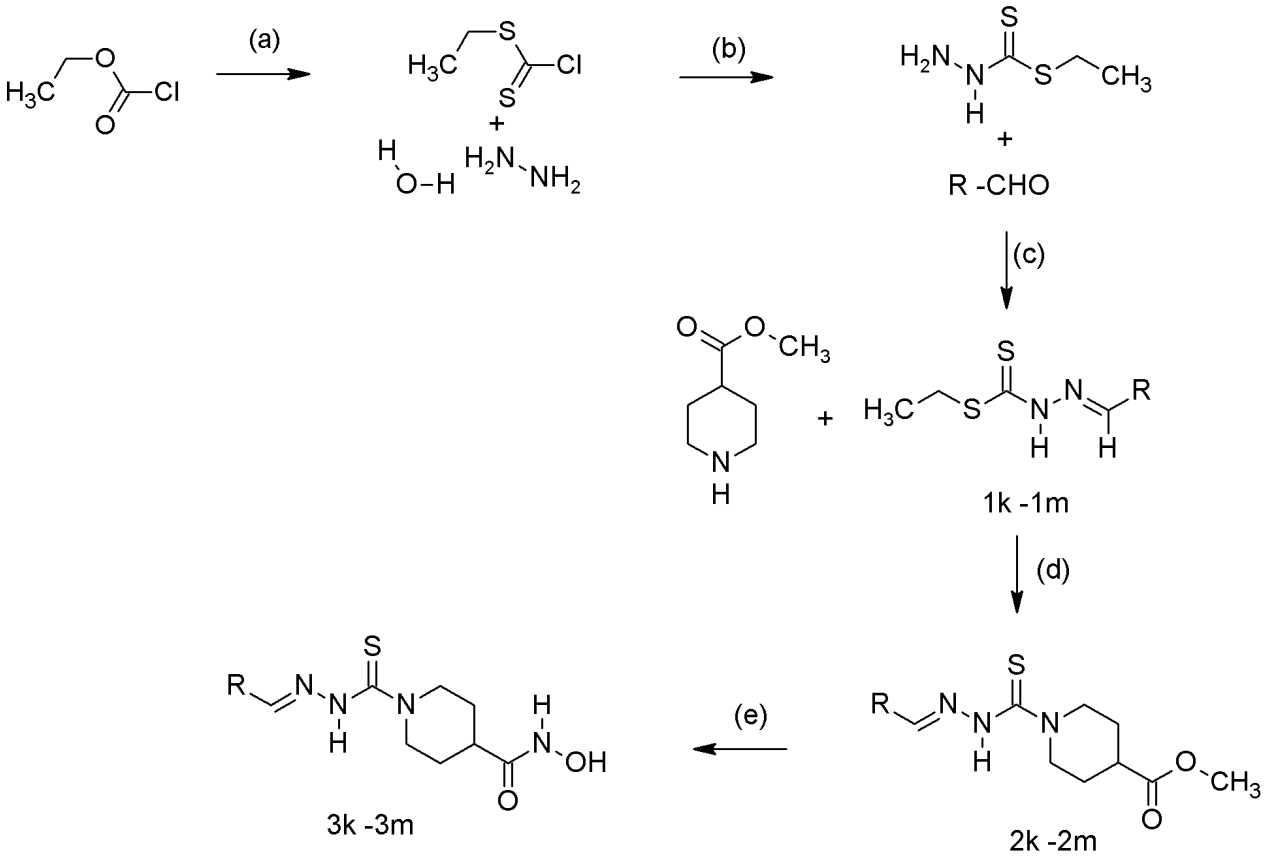


Reaction& condition:

(a) CH_3OH, (C_2H_5) N, (3hr stirring); (b) CH_3OH, CH_3COOH (2-3hr reflux);

(c) Metylpiperidine-4-corboxylate, CH_3OH, (6-8 hrs reflux); (d) K2CO3, $(C2H5)_2$ O, NH2OH.HCl, (1-2 hrs stirring).

Scheme 2



Reaction& condition:

(a)CH_3OH, (C_2H_5) N, (3hr stirring); (b) Lawesson's reagent, toluene, (2hrs reflux); (c) CH_3OH, CH_3COOH (2-3hr reflux); (d) Metylpiperidine-4-corboxylate, CH_3OH, (6-8 hrs reflux); (e) K_2CO_3, $(C_2H_5)_2$ O; $NH_2OH.HCl$, (1-2 hrs stirring).

Phase-2

Characterization:

All the newly synthesized compounds will be characterized by Melting point determination; Solubility property, TLC analysis and their structure will be elucidated by UV Spectroscopy, IR-Spectroscopy, H^1NMR –Spectroscopy and MASS-Spectroscopy.

Phase-3

Biological evaluation:

***In vitro* anticancer screening**

All synthesized compounds would subject for *in vitro* anticancer activity on the cervical cancer cell line (HeLa)..

3.2 DOCKING ANALYSIS[8]:

Compounds 1-53 were used for docking on 1 cancer protein histone deacetylase (HDAC) PDB ID: 1T69.

Glide docking uses the assumption of a rigid receptor, although scaling of van der Waals radii of nonpolar atoms, which decreases penalties for close contacts, can be used to model a slight "give" in the receptor and/or ligand. Docking studies of designed compounds were carried out using GLIDE (Grid-based Ligand Docking with Energetics) module version 4.5, Schrödinger, LLC, New York, NY, 2007. The software package running on multi-processor Linux PC. GLIDE has previously been validated & applied successfully to predict the binding orientation of many ligands.

DOCKING METHODOLOGY

The steps involved in docking are as follows:

- **Ligand structure**: The chemical structure of each ligand was drawn using build module**.**
- **Ligand preparation**: In order to prepare high quality, all-atom 3D structures for large numbers of drug-like molecules, starting with the 3D structures in SD Maestro format, LigPrep was used. LigPrep produced a single, low-energy, 3D structure with corrected chiralities for each successfully processed input structure.

- **Preparation of protein**: The typical structure file from the PDB is not suitable for immediate use in molecular modelling calculations. A typical PDB structure file consists only of heavy atoms and may include a co-crystallized ligand, water molecules, metal ions, and cofactors. Some structures are multimeric, and may need to be reduced to a single unit. Because of the limited resolution of X-ray experiments, it can be difficult to distinguish between NH and O, and the placement of these groups must be checked. PDB structures may be missing information on connectivity, which must be assigned, along with bond orders and formal charges. This was done using the Protein Preparation Wizard.
- **Receptor Grid Generation**: Receptor grid generation requires a "prepared" structure: an all atom structure with appropriate bond orders and formal charges. Glide searches for favourable interactions between one or more ligand molecules and a receptor molecule, usually a protein. The shape and properties of the receptor are represented on a grid by several different sets of fields that provide progressively more accurate scoring of the ligand poses. The options in each tab of the Receptor Grid Generation panel allow defining the receptor structure by excluding any co-crystallized ligand that may be present, determine the position and size of the active site as it will be represented by receptor grids, and set up Glide constraints. A grid area was generated around the binding site of the receptor.
- **Ligand Docking**: This is carried out using GLIDE DOCK. Glide searches for favourable interactions between one or more ligand molecules and a receptor molecule, usually a protein. Each ligand acts as single molecule, while the receptor may include more than one molecule, e.g., a protein and a cofactor. Glide was run in rigid or flexible docking modes; the latter automatically generated conformations for each input ligand. The combination of position and orientation of a ligand relative to the receptor, along with its conformation in flexible docking, is referred to as a ligand pose. The ligand poses that Glide generates pass through a series of hierarchical filters that evaluate the ligand's interaction with the receptor. The initial filters test the spatial fit of the ligand to the defined active site, and examine the complementarity of ligand-receptor interactions using a grid-based method patterned after the empirical ChemScore function. Poses that passed these initial screens entered the final stage of the algorithm, which involves evaluation and minimization of a grid approximation to the OPLS-AA non bonded

ligand-receptor interaction energy. Final scoring is then carried out on the energy-minimized poses.

- **Glide Extra-Precision Mode (XP)-**The extra-precision (XP) mode of Glide combines a powerful sampling protocol with the use of a custom scoring function designed to identify ligand poses that would be expected to have unfavourable energies, based on well-known principles of physical chemistry. The presumption is that only active compounds will have available poses that avoid these penalties and also receive favourable scores for appropriate hydrophobic contact between the protein and the ligand, hydrogen-bonding interactions, and so on. The chief purposes of the XP method are to weed out false positives and to provide a better correlation between good poses and good scores. Extra-precision mode is a refinement tool designed for use only on good ligand poses. Finally, the minimized poses are re-scored using Schrödinger's proprietary *GlideScore* scoring function. GlideScore is based on ChemScore, but includes a steric-clash term and adds buried polar terms devised by Schrodinger to penalize electrostatic mismatches:

Glide Score = 0.065*vdW + 0.130*Coul + Lipo + Hbond + Metal + BuryP + RotB + Site

Table: 3 Components of Glide Score of Docking (Extra-Precision Mode)

Component	Description

VdW	Van der Waals energy is calculated with reduced net ionic charges on groups with formal charges, such as metals, carboxylates, and guanidiniums.
Coul	Coulomb energy is calculated with reduced net ionic charges on groups with formal charges, such as metals, carboxylates, and guanidiniums.
Lipo	Lipophilic contact term. Rewards favourable hydrophobic interactions.
HBond	Hydrogen-bonding term is separated into differently weighted components that depend on whether the donor and acceptor are neutral, one is neutral and the other is charged, or both are charged.
Metal	Metal-binding term used only for the interactions with anionic acceptor atoms is included. If the net metal charge in the apo protein is positive, the preference for anionic ligands is included; if the net charge is zero, the preference is suppressed.
BuryP	Penalty for buried polar groups.
RotB	Penalty for freezing rotatable bonds.
Site	Polar interactions in the active site. Polar but non-hydrogen-bonding atoms in a hydrophobic region are rewarded.

Docking Procedure:

The computational modeling studies relied upon the GLIDE (Grid-based Ligand Docking from Energetics) program (Glide, version 5.0, Schrodinger, LLC

New York, 2008) for the docking simulations. These simulations were performed using the X-ray crystal structure of the human HDAC 8 complexed with SAHA (PDB ID: 1T69). All the water molecules in the crystal structure were deleted, bond orders were assigned, hydrogen's were added and the protein was then further refined for the docking studies by processing it using Schrodinger's Protein preparation wizard. This procedure minimizes the protein to 0.30 Å RMSD using OPLS-2001 force field. Ligands were prepared using build panel in maestro. Further the ligands were prepared for docking using LigPrep tool and were energy minimized using MMFF Force Field. Glide Grid generation panel has been used to generate receptor grid for docking. Default SP (Standard Precision) docking protocol was used to dock the library ligands.

Molecular docking were performed for 53 compounds using the GLIDE program (Version 5.0, Schrodinger, LLC, New York, 2008) to understand the interaction of 3k with HDAC8. The Maestro user interface (version8.5, Schrodinger, LLC, New York, 2008) was employed to set up and execute the docking protocol and also for analysis of the docking results. Human HDAC8 (PDB ID: 1T69) was selected for docking studies and was prepared for docking through protein preparation wizard, energy minimization has been carried out using OPLS2001 force field. Structures of 1a-1m were sketched using built panel on Scheme 1&2. Maestro and prepared for docking through Ligprep module (energy minimized using MMFF force field). GLIDE grid generation wizard has been used to define the docking space. Docking was performed using SP (Standard Precision mode) docking protocol. The molecular docking results are presented in **Table 2**. ZBG hydroxamate functional group of all the molecules were found to be close to Zn2+ atom in the active site, and establishes a hydrogen bond with TRP 137 and GLY139 which shows the major and favorable interaction of the ligands with HDAC8 **(Fig.3l)**. Amongst the nine molecules docked, compound 3l was the one with the best Glide and E model score of (-8.30 & 92.2) respectively. It exhibited two hydrogen bonding interaction with TRP 137 and GLY139 (Fig. 3k). The hydroxamate group is placed near the Zn2+ atom.

Table: 4

MOLECULAR DOCKING

CODE	GLIDE SCORE	E MODEL SCORE
E14	-9.6014	-89.7972
E8	-8.3436	-92.9220
3l	-8.2066	-60.5982
E1	-7.8502	-60.3135
E14	-7.3526	-83.6240
E3	-5.9720	-39.3396
3k	-5.8887	-0.5451
E3	-4.8042	37.6200
E7	-2.7761	-14.2221

Snapshots of drug-receptor interaction

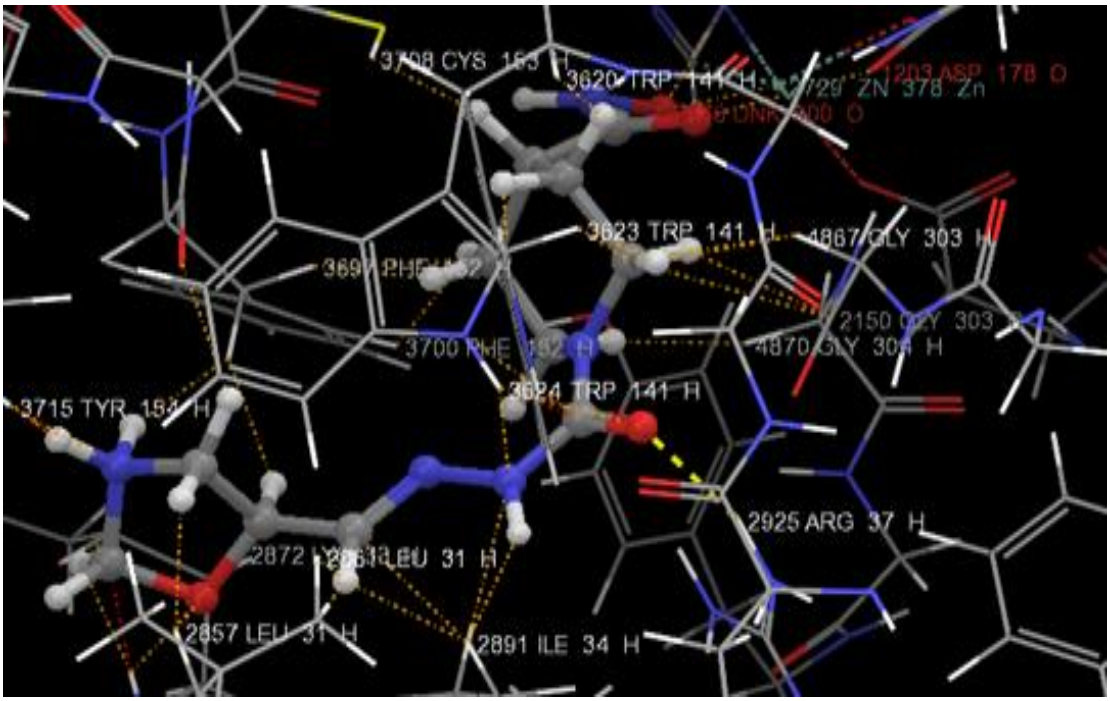


Fig .5 Interactions of (E14) active site residues with 1T69

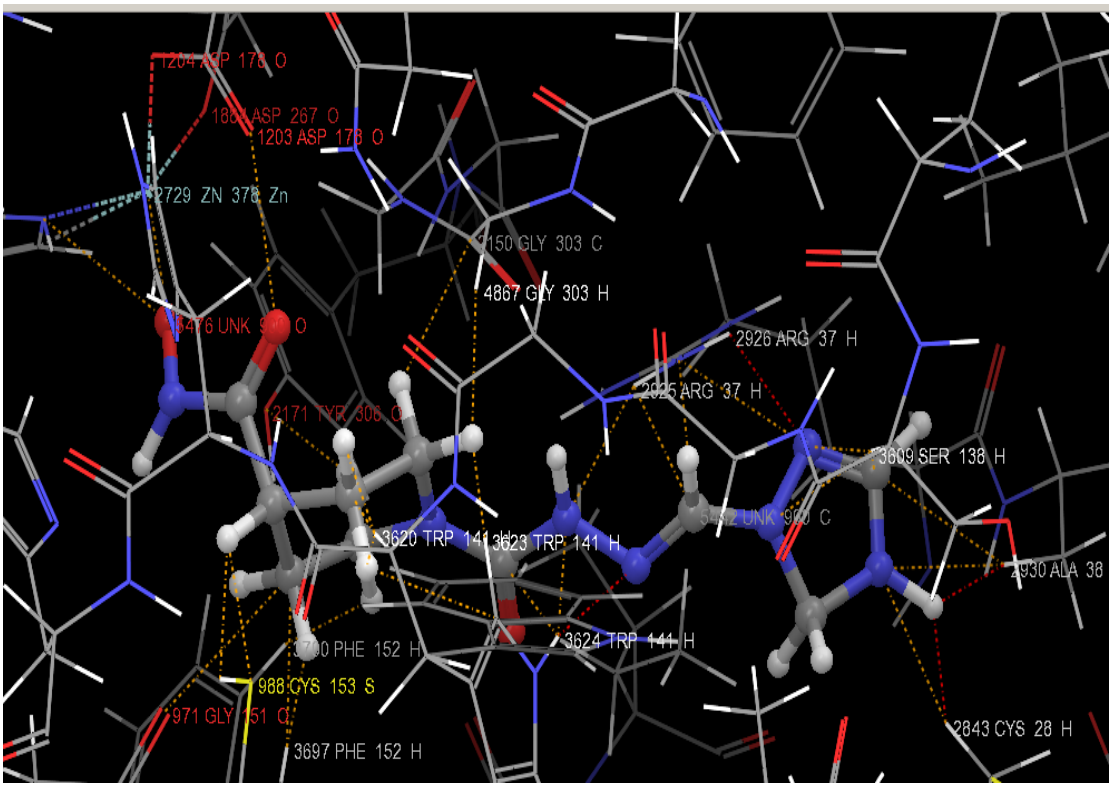


Fig .6 Interactions of (E8) active site residues with 1T69

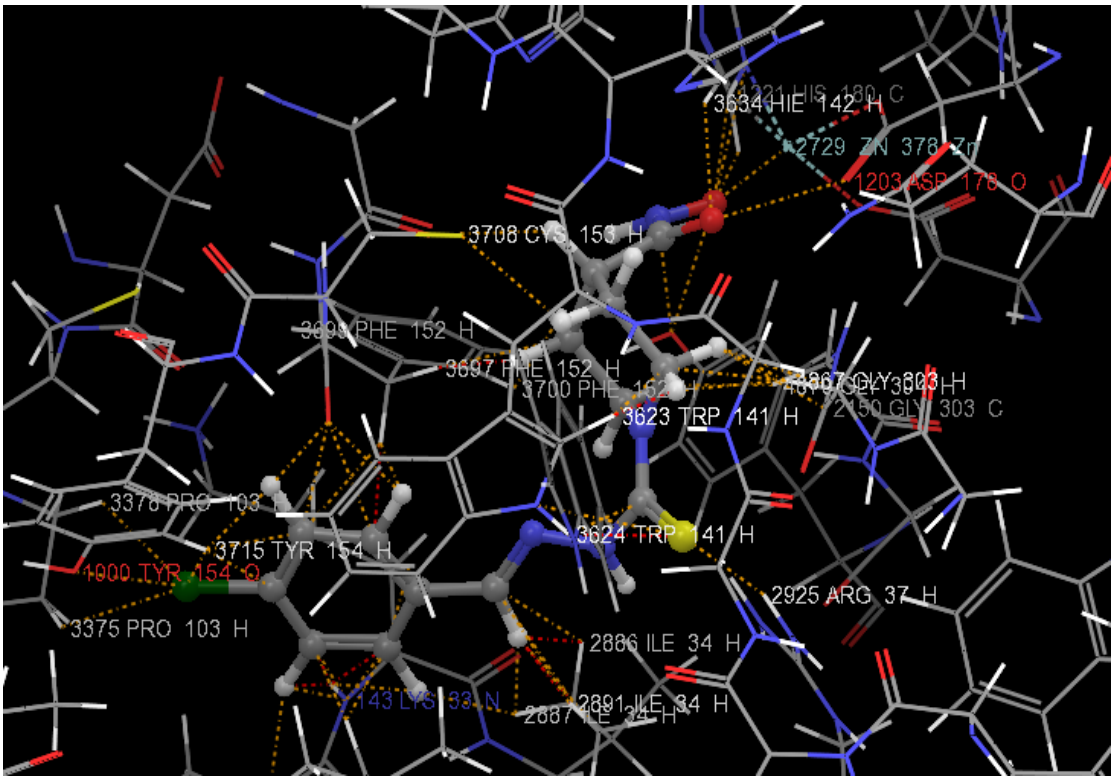


Fig.7 Interaction of (3k) active site residues with 1T69

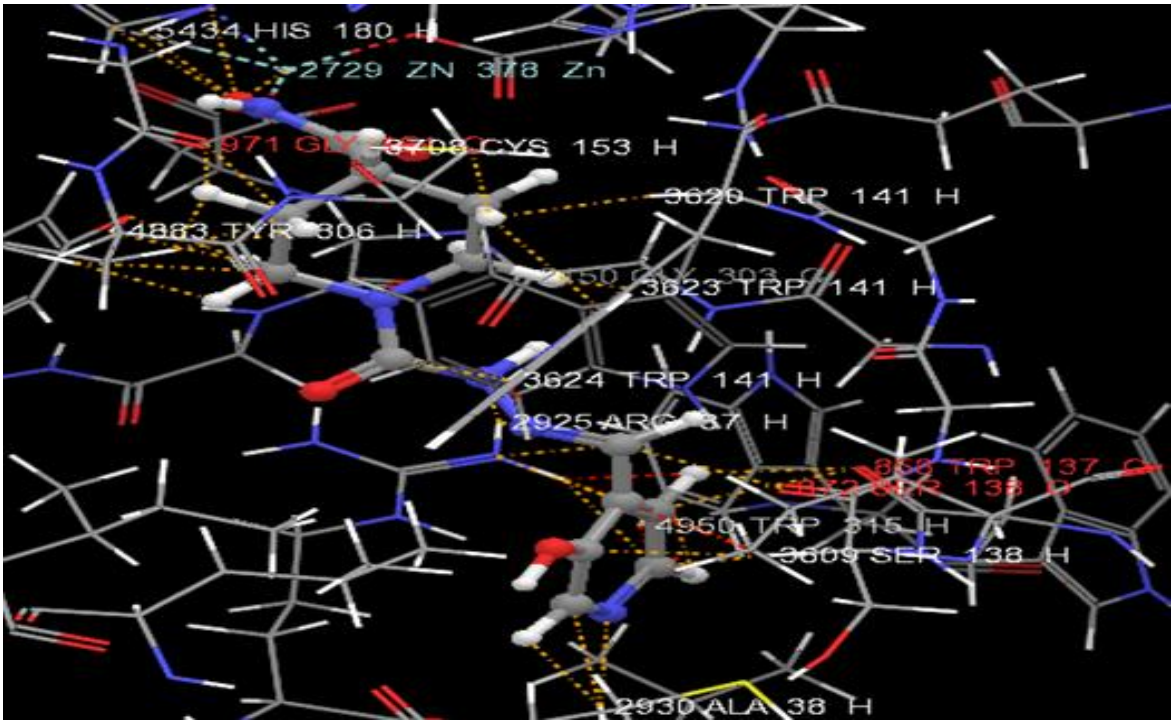


Fig.8 Interaction of (E1) active site residues with 1T69

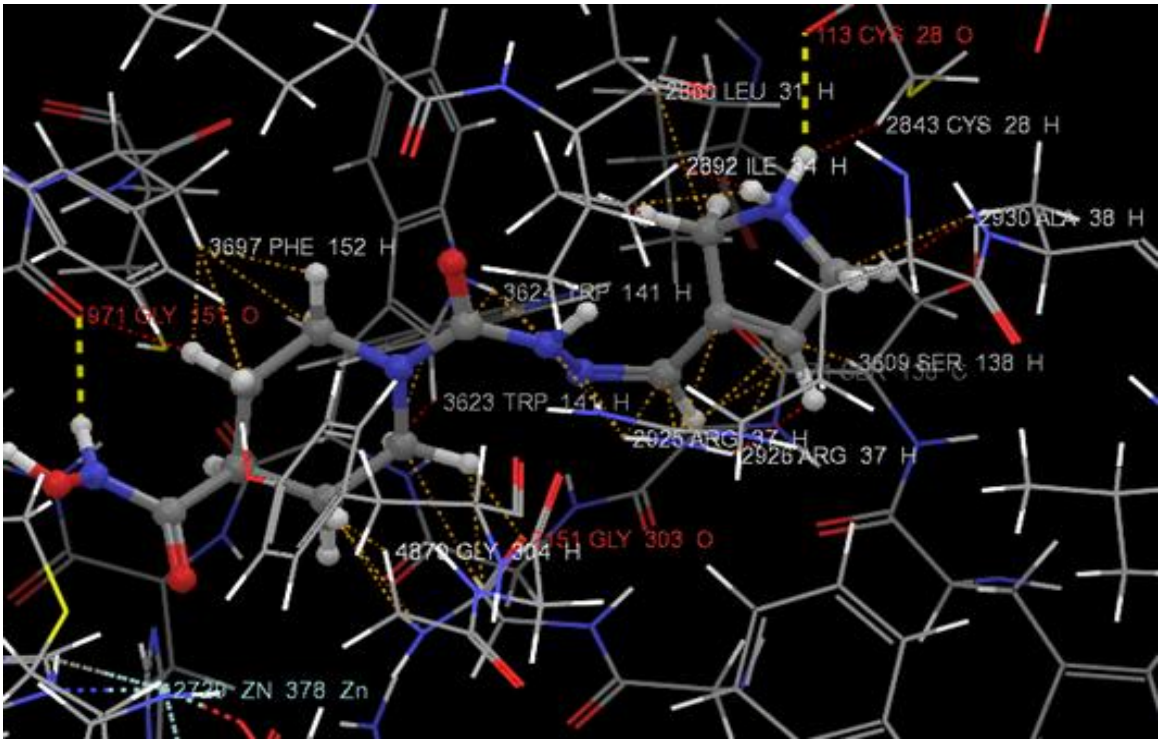


Fig .9 Interactions of (E3) active site residues with 1T69

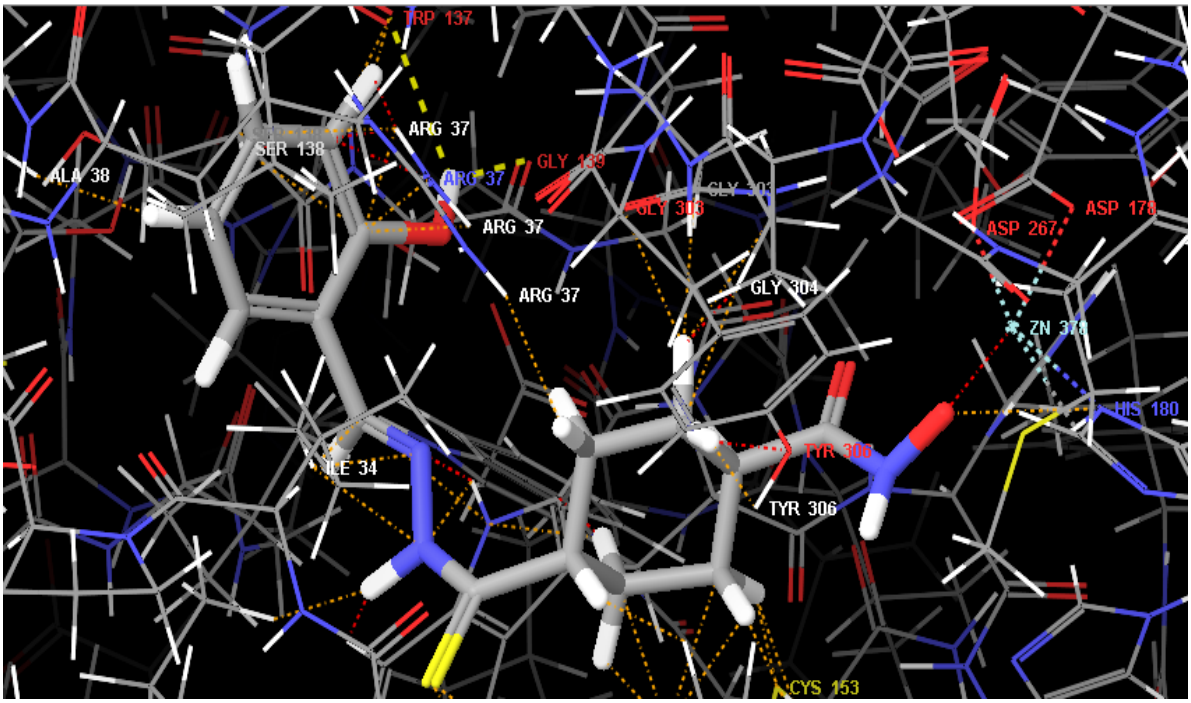


Fig .10 Interactions of (3l) active site residues with 1T69

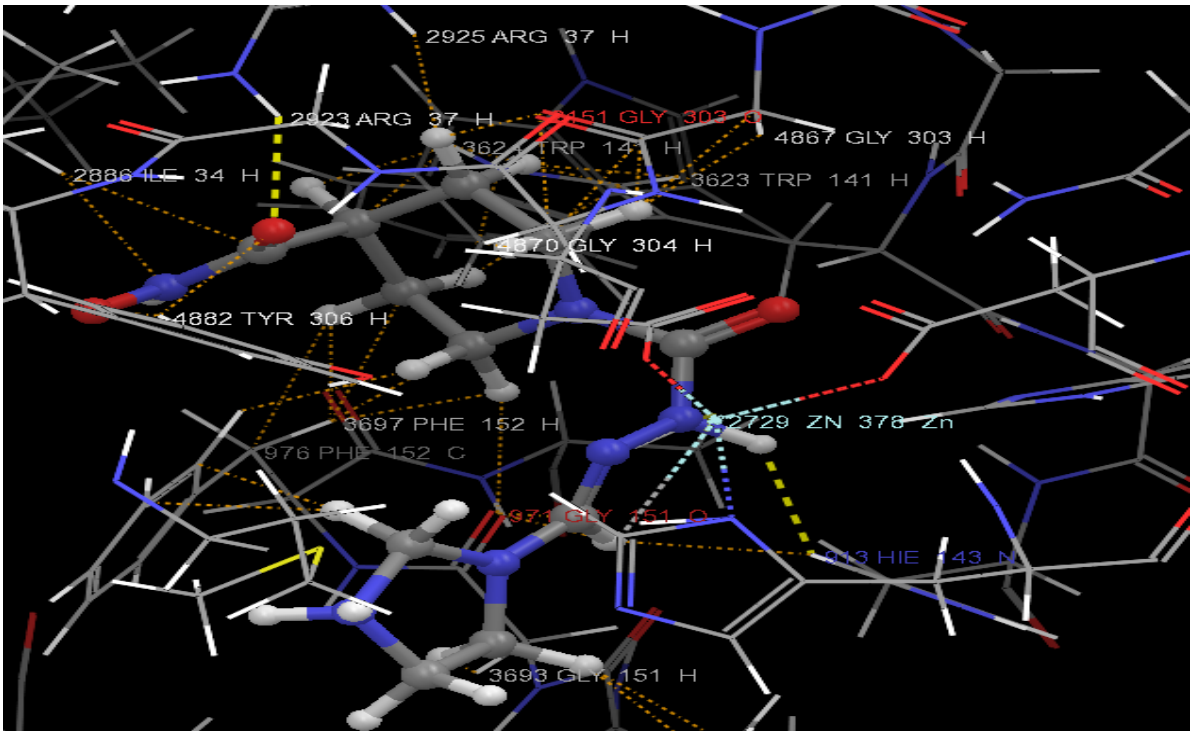


Fig .11 Interactions of (E7) active site residues with 1T69

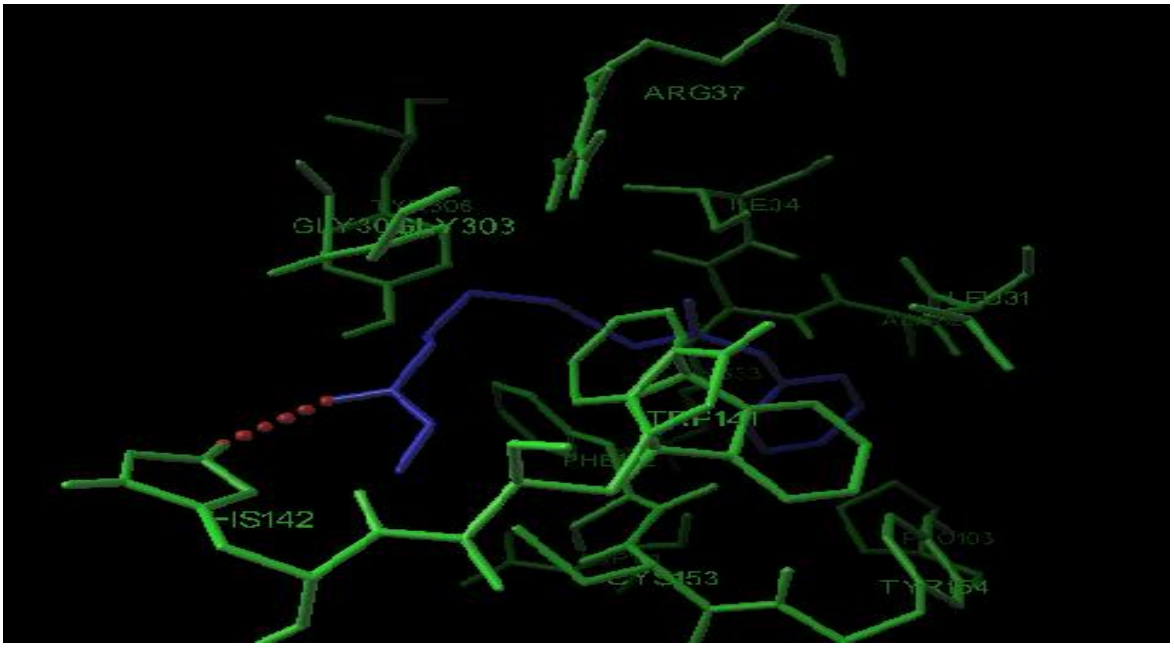


Fig .12 Interactions of (SAHA) active site residues with 1T69 in HIS 142

Validation of docking protocol was done by redocking. Human HDAC8 bound to SAHA (PDB ID: 1T69) was selected for docking studies by using autodock version 4.0

- Ligand constructed by tube representation
- Hydrogen bond interaction is representated by yellow broken lines
- Green colour representated protien human HDAC8 ID:1T69
- Blue colour representated SAHA standard drug (HDAC) inhibitor
- Red dotted line indicated hydrogen bond inraaction

- **E14** is best glide score and E model score (-9.6014 & -89.7972) respectively. ZBG hydroxamate functional group of the molecule were found to Zn^{2+} atom in the active site, and establishes a hydrogen bond interaction with 2925 ARG 37 and shows the major favourable interaction of ligand with HDAC.
- **E8** is one of the best glide score and E model score (8.3436 &-92.9220) respectively. ZBG hydroxamate functional group of the molecule were found to Zn^{2+} atom in the active site, and establishes a no hydrogen bond interaction with HDAC.
- **3k** is one of the best glide score and E model score (-8.2066 &-60.5982) respectively. ZBG hydroxamate functional group of the molecule were found

to Zn^{2+} atom in the active site, and establishes a hydrogen bond interaction with (858 TRP137 O&GLY137) and shows the major favourable interaction of ligand with HDAC.

- **E1** is one of the best glide score and E model score (-7.8502&-60.3135) respectively. ZBG hydroxamate functional group of the molecule were found to Zn^{2+} atom in the active site, and establishes a no hydrogen bond interaction ligand with HDAC.
- **E3** is one of the best glide score and E model score (-5.9720&-39.3396) respectively. ZBG hydroxamate functional group of the molecule were found to Zn^{2+} atom in the active site, and establishes a hydrogen bond interaction with (113CYS 280&971GLY 151 O) and shows the major favourable interaction of ligand with HDAC.
- **3l** is one of the best glide score and E model score (-5.8887&-0.5451) respectively. ZBG hydroxamate functional group of the molecule were found to Zn^{2+} atom in the active site, and establishes a no hydrogen bond interaction with HDAC.
- **E7** is one of the best glide score and E model score (-2.7761&-14.2221) respectively. ZBG hydroxamate functional group of the molecule were found to Zn^{2+} atom in the active site, and establishes a hydrogen bond interaction with (HIE 143&ARG37) and shows the major favourable interaction of ligand with HDAC

3.3 EXPERIMENTAL

General Procedure:

Scheme 1

Step 1

General procedure for preparation of ethyl hydrazinecarboxylate: [2](1)

An equimolar quantities of hydrazine hydrate (0.1mol, 0.52ml) and ethyl chloroformate (0.1mol, 9.4 ml) and triethyl amine (0.1mol, 14.27 ml) and were dissolved in methanol and stirred for 2 hours under room temperature. A white precipitate was separated, filtered and dried in a vacuum to yield ethyl hydrazinecarboxylate

Step 2

General procedure for the synthesis of Schiff's base of Ethyl 2- substituted methylidene hydrazine carboxylate[2] (1a-j):

Ethyl hydrazino formate (1.04g, 0.01mol) and substituted benzaldehydes (0.01mol) were dissolved in methanol and maintained the pH 4-5 using glacial acetic acid. It was refluxed for 1-3 hours and the reaction was monitored by thin layer chromatography using (methanol: chloroform) 1:1 as mobile base. After completion, the reaction mixture was poured into crushed ice and precipitate was filtered and recrystalized from methanol, to yield a pure compound as a green crystals of Ethyl 2-substituted methylidene hydrazine carboxylate.

Step 3

General procedure for synthesis of substituted methyl 1-(ethylidine aminocarbamoyl) piperidine -4-carboxylate[2] (2a-j):

An eqimolar quqntities of methyl piperidine – 4 –carboxylate (1.34ml, 0.01mol) and Ethyl 2- substituted methylidene hydrazine carboxylate (0.01mol) were dissolved in methanol and refluxed for 6-8 hours. The reaction was monitored by TLC using (methanol: chloroform) 1:1. Then the reaction mixture was poured into crushed ice. The precipitate was filtered and recrystalized from methanol or chloroform to yield a pure compound as a green crystals of substituted methyl 1-(ethylidine aminocarbamoyl) piperidine -4-carboxylate(2a-2j).

Step 4

General procedure for synthesis of substituted 4-(hydroxycarbamoyl)-N'-ethylidenepiperidine-1-carbohydrazide[1] (3a-j):

Substituted 4-(hydroxycarbamoyl)-N'-ethylidenepiperidine-1-carbohydrazide was synthesized by using modified procedures of Grobner and Steinberg. Equimolar quantity of (0.06 mol) of substituted methyl 1-(ethylidine aminocarbamoyl) piperidine -4-carboxylate was added to the mixture of hydroxylamine hydrochloride (0.06mol) and potassium carbonate (0.06mol) in moist ether. The above solution was allowed to cool at 10^0C and stirred for 1-2 hrs and an excess solvent was evaporated at room temperature and recrystalized to yield a substituted 4-(hydroxycarbamoyl)-N'-ethylidenepiperidine-1-carbohydrazide.

Scheme 2

Step 1

General procedure for preparation of ethyl carbonochloridodithioate[1] (II)

Ethyl chloroformate (0.1mol, 9.4 ml) and (0.2mol, 8.08gm lawessons reagent are dissolved in toluene (20 ml) and refluxed for 2 hours and the reaction completion was monitored by thin layer chromatography using (methanol: chloroform) 1:1.

Step 2

General procedure for preparation of ethyl hydrazinecarbodithioate[1] (III)

An equimolar quantities of hydrazine hydrate (0.1mol, 0.52ml) and ethyl carbonochloridodithioate and triethyl amine (0.1mol, 14.27 ml) and were dissolved in methanol and stirred for 2 hours under room temperature. A white precipitate was separated, filtered and dried in a vacuum to yield ethyl carbonochloridodithioate.

Step 3

General procedure for synthesis of substituted Schiff's base of ethyl 2-substituted methylidene hydrazine carbothioate[2] (1k-m)

Ethyl hydrazinecarbothioate (1.04g, 0.01mol) and substituted benzaldehyde (0.01mol) were dissolved in methanol and maintained the pH 4-5 using glacial acetic acid. It was refluxed for 1-3 hours and the reaction completion was monitored by thin layer chromatography using (methanol: chloroform) 1:1. After completion reaction was poured into crushed ice and the precipitate was filtered and recrystalized using methanol, to yield a pure compound as a green crystals of ethyl 2- substituted methylidene hydrazine carbothioate.

Step 4

General procedure for synthesis of substituted methyl (1-methyleneamino thiocarbomyl) piperidine -4-corboxylate[2] (2k-m)

An eqimolar quqntities of methyl piperidine – 4 –corboxylate (1.34ml, 0.01 mol) and ethyl 2- substituted methylidene hydrazine carbothioate were dissolved in methanol and refluxed for 6-8 hours. The reaction condition was monitored by thin layer chromatography using (methanol: chloroform) 1:1. Immediately the reaction mixture was poured into crushed ice. The precipitate was filtered and recrystalized using methanol, or chloroform to yield a pure compound as a green crystals of substituted methyl (1-methyleneamino thiocarbomyl) piperidine -4-corboxylate (2k-2l).

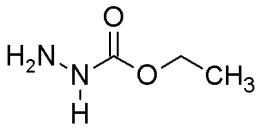
Step 5

General procedure for synthesis of substituted *N*-hydroxy-1-[(2- methylidene hydrazinyl) carbonothioyl] piperidine-4-carboxamide[1] (3k-m)

Substituted *N*-hydroxy-1-[(2- methylidene hydrazinyl) carbonothioyl] piperidine-4-carboxamide carbohydrazide was synthesized by using modified procedures of Grobner and Steinberg. Equimolar quantity of (0.06mol) substituted methyl (1-methyleneamino thiocarbomyl) piperidine -4-corboxylate was added to the mixture of hydroxylamine hydrochloride (0.06mol) and potassium carbonate (0.06mol) in moist ether and allowed to cool at 10^0C. Then the whole solution was Stirred for 1-2 hrs. The precipitate was filtered.

Scheme 1

Step 1

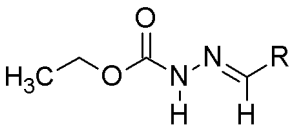


(I)

Table. 5

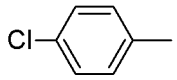
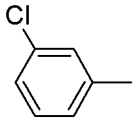
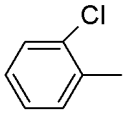
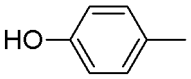
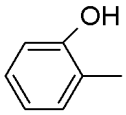
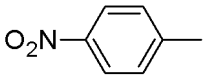
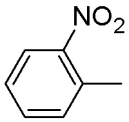
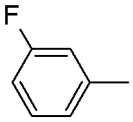
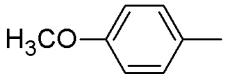
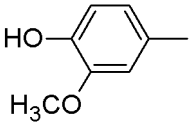
Code	M.F	M .W	Yield (%)	R_f	Colour	Solubility
1	$C_3H_8N_20_2$	104.10	91	0.77	White	Methanol

Step 2



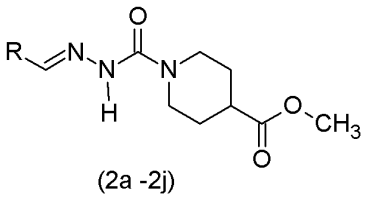
1a -1j

Table. 6

Code	R	M.F	M .W	M .P	Yield (%)	ClogP
1a		$C_{10}H_{10}N_2O_2Cl$	226.66	230-235	44	2.99
1b		$C_{10}H_{10}N_2O_2Cl$	226.66	240-250	42	2.99
1c		$C_{10}H_{10}N_2O_2Cl$	226.66	130-135	33	2.99
1d		$C_{10}H_{12}N_2O_3$	208.21	270-280	48	1.61
1e		$C_{10}H_{12}N_2O_3$	208.21	220-225	43	1.61
1f		$C_{10}H_{11}N_3O_3$	237.21	305-310	46	2.02
1g		$C_{10}H_{11}N_3O_3$	237.21	205-210	53	2.02
1h		$C_{10}H_{11}N_2O_2F$	210.20	135-140	45	2.42
1i		$C_{10}H_{14}N_2O_3$	222.24	185-190	85	2.19
1j		$C_{11}H_{14}N_2O_4$	238.36	245-250	82	1.46

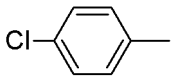
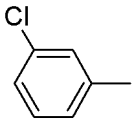
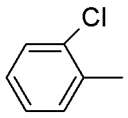
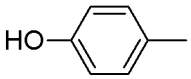
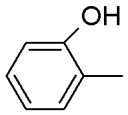
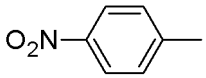
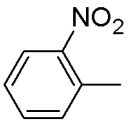
*All compounds are freely soluble in DMSO & partially soluble in chloroform

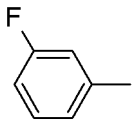
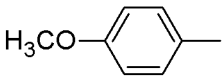
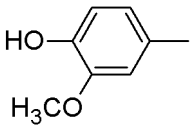
Step 3



(2a -2j)

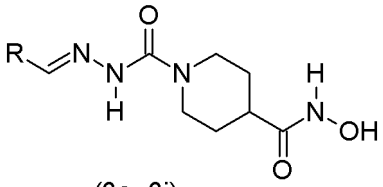
Table. 7

Code	R	M.F	M .W	M .P	Yield (%)	ClogP
2a		$C_{15}H_{18}N_3O_3Cl$	323.77	200-205	92	2.84
2b		$C_{15}H_{18}N_3O_3Cl$	323.07	153-156	71	2.84
2c		C1518N3O3CL	323.07	126-129	70	2.84
2d		$C_{15}H_{19}N_3O_4$	305.33	318-320	66	1.46
2e		$C_{15}H_{19}N_3O_4$		248-253	55	1.46
2f		$C_{15}H_{18}N40_5$	334.13	330-335	86	1.87
2g		$C_{15}H_{18}N_40_5$	334.13	227-231	64	1.87

Code	R	M.F	M .W	M .P	Yield (%)	ClogP
2h		$C_{14}H_{18}N_3O_2F$	307.32	140-143	68	2.27
2i		$C_{16}H_{21}N_3O_4$	319.36	176-179	93	2.04
2j		$C_{16}H_{21}N_3O_5$	335.36	231-233	96	1.31

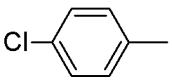
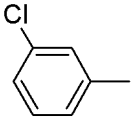
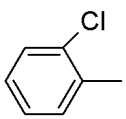
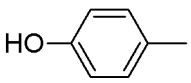
*All compounds are freely soluble in DMSO & partially soluble in chloroform

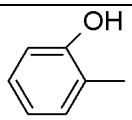
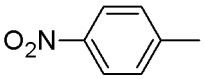
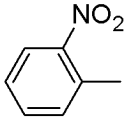
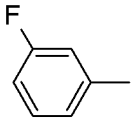
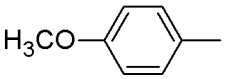
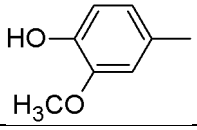
General structure



(3a -3j)

Table.8

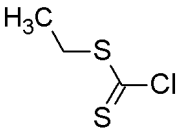
Code	R	M.F	M .W	M .P	Yield (%)	ClogP
3a		$C_{14}H_{17}N_4O_3Cl$	324.76	230	51	1.06
3b		$C_{14}H_{17}N_4O_3Cl$	324.76	205-215	56	1.06
3c		$C_{14}H_{17}N_4O_3Cl$	324.76	105	43	1.06
3d		$C_{14}H_{18}N_4O_4$	306..32	280	24	-0.31

Code	R	M.F	M .W	M .P	Yield (%)	ClogP
3e		$C_{14}H_{18}N_4O_4$	306.32	240	32	-0.31
3f		$C_{14}H_{17}N_5O_5$	335.32	340	34	0.09
3g		$C_{14}H_{17}N_5O_5$	335.32	220	70	0.09
3h		$C_{14}H_{17}FN_4O_3$	308.13	126-130	56	0.49
3i		$C_{15}H_{20}N_4O_4$	320.34	186-190	86	0.27
3j		$C_{15}H_{20}N_4O_5$	336.34	220	67	0.46

*All compounds are dissolving in DMSO & partial soluble in chloroform

Scheme 2

Step 1

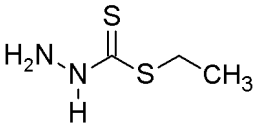


(II)

Table.9

Code	M.F	M .W	Yield (%)	R_f	Colour	Solubility
	$C_3H_5ClS_2$	140.65	86	0.60	colourless	-

Step 2

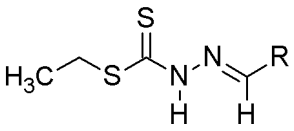


(III)

Table-10

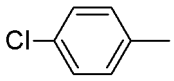
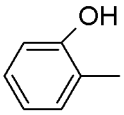
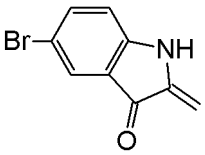
Code	M.F	M .W	Yield (%)	R_f	Colour	Solubility
III	$C_3H_8N_2S_2$	136.23	95	0.76	White	Methanol

Step 3



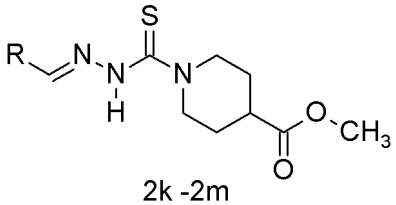
1k -1m

Table.11

Code	R	M.F	M .W	M .P	Yield (%)	ClogP
1k		$C_9H_9S_2N_2Cl$	244.76	180-190	46	3.39
1l		$C_{10}H_{12}N_2OS_2$	224.06	220-225	65	1.61
1m		$C_{11}H_{10}N_3OS_2$	334.25	300-310	56	3.35

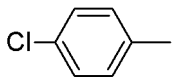
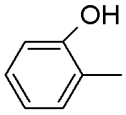
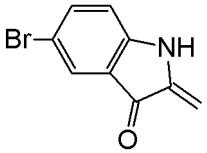
*All compounds are dissolving in DMSO & partial soluble in chloroform

Step 4



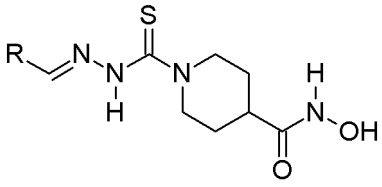
2k -2m

Table.12

Code	R	M.F	M .W	M .P	Yield (%)	ClogP
2k		$C_{15}H_{18}N_3O_2SCl$	339.84	213-216	43	2.99
2l		$C_{15}H_{18}N_3O_3S$	329.39	356-370	43	1.61
2m		$C_{16}H_{17}N_4O_4SBr$	423.30	335-342	87	2.58

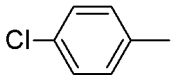
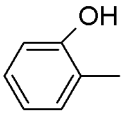
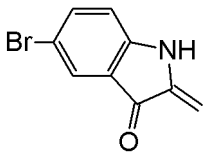
*All compounds are dissolving in DMSO & partial soluble in chloroform

Step 5



3k -3m

Table.13

Code	R	M.F	M .W	M .P	Yield (%)	ClogP
3k		$C_{14}H_{17}N_4O_2Cl$	340.82	265-269	76	1.12
3l		$C_{14}H_{17}ClN_4O_3s$	322.38	230	54	-0.16
3m		$C_{15}H_{26}N_5O_4SBr$	442.23	220-225	96	1.34

*All compounds are dissolving in DMSO & partial soluble in chloroform

3.3. ANALYTICAL WORK

THE LAYER CHROMATOGRAPHY

Thin layer Chromatography is a method of analysis in which the stationary phase, a finely divided solid, is spread as a thin layer on a rigid supporting plate and the mobile phase, a liquid is allowed to migrate across the surface of the plate.

Applications of TLC

1. To establish the purity and authenticity of starting materials and reagents.
2. To monitor the reactions, particularly in the case of new reactions.
3. Assessment of purity of a crude reaction product.
4. The optimum of experimental conditions to achieve the highest possible yield of product.

Provided that the experimental conditions are reproducible, the movement of any substance relative to the solvent front in a given chromatographic system is constant and characteristic of the substance. The constant is called as retardation factors (R_f) and is defined as

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance traveled by the solvent front}}$$

True reproducibility in R_f value is, however, rarely achieved in practice due to minor changes in a number of variables such as:

1. The particle size of different batches of adsorbent.
2. The solvent composition and the degree of saturation of the chamber atmosphere with solvent vapor.
3. Prior activation and storage conditions of the plates.
4. The thickness of adsorbent layer, etc.

It is therefore, not desirable to use R_f value in isolation as a criteria for identity.

TLC were performed as following procedure

Dimension of plates	:	5×20cm
Stationary phase	:	Silica gel-G 9E-merck)
Mobile phase	:	Chloroform: Methanol (1:1)
Technique	:	Ascending
Detection Method	:	Iodine chamber

Preparation of Plates

Uniform slurry of silica get G was prepared by addition of distilled water. This was then poured into a spreading through and drawn across a series of glass plates of size 5×20cm, depositing a uniform layer of stationary phase of 0.25mm thickness. The plates were air dried and then activated by heating at 110 ^{0}C for one hour. The plates were stored over a dessicator until used.

Mobile Phase

Evaluation of various mobile phases was tried, alone or in combination for each compound in which Chloroform: Methanol (1:1) was found to be suited.

Sample Application and Development

The samples were applied as small spot at about 2cm form the base of the plate. For ascending development of the thin layer chromatogram, the plate was placed in a TLC Chamber, which was saturated with mobile phase containing the developing solvent to a depth of about 0.5cm. The solvent was allowed to move up the plate until it travelled a distance of about 15cm from the point of the sample, on a 20cm plate. The plate was then removed from the chamber, the solvent front was marked by scratching the surface and the plate was allowed to be evaporated

Detection

After the chromatogram was developed the solute sports need to be made visible in order to determine their R_f values. Iodine chamber was employed for the

detection of the compounds by placing the plate in iodine chamber containing iodine crystals .The solutes were visible as amper colour spots.

Table.15

Code	R_f	Code	R_f	Code	R_f
1a	0.74	2a	0.35	3a	0.70
1b	0.70	2b	0.38	3b	0.62
1c	0.64	2c	0.36	3c	0.81
1d	0.74	2d	0.75	3d	0.68
1e	0.60	2e	0.45	3e	0.74
1f	0.64	2f	0.60	3f	0.67
1g	0.36	2g	0.80	3g	0.85
1h	0.62	2h	0.38	3h	0.76
1i	0.40	2i	0.70	3i	0.90
1j	0.45	2j	0.80	3j	0.82
1k	0,70	2k	0.63	3k	0.70
1l	0.36	2l	0.54	3l	0.66
1m	0.55	2m	0.77	3m	0.60

3.4. INFRARED SPECTRAL STUDY

The range of electromagnetic radiation between 0.8 and 500µm is referred as infrared radiation. The IR spectrum is represented with percent transmittance as the ordinate and the wave number (cm-1) as the abscissa. The most commonly used region of IR spectrum in pharmaceutical chemistry is between 2.5µm (400cm-1) and 16µm (625cm-1). Two major applications of IR spectrometry are characterization of

Various molecules include Determination of identity of a compound by means of spectral comparison with that of authentic sample.

Verification of the presence of functional groups in unknown molecules which is quite important in the structural elucidation of synthetic organic compounds or substances isolated from natural sources.

Samples were prepared by KBr Disc method. Solid samples (0.5-1.Omg) were intimately mixed with powdered potassium bromide. Mixing was effected through

grinding in a smooth agate mortar and the mixture pressed between a punch and disc under the presence of 1, 00,000-15,000 psi into a transparent disk.

- The infrared spectral study was done on JASCO FTIR 4100.
- The spectral data are given as follows.All absorption values are expressed in wave numbers cm^{-1}

Table. 16

CODE	ABSORBANCE cm^{-1}	GROUPS
3a	3446.42	Secondary amine -N-H stretch
	3200.98	Hydroxyl-O-H stretch
	3050.00	Aromatic -C-H stretch
	2901.02	Methylene -C-H stretch
	1642.09	Imino -C=N- stretch
3b	3250.03	Secondary amine -N-H stretch
	3050.04	Aromatic -C-H stretch
	2930.01	Methylene -C-H stretch
	1690.04	Amide -C=O stretch
	1630.10	Imino -C=N- stretch
	1627.01	Aromatic -C=C- stretch
	1100.02	Aromatic -C-Cl stretch
3c	3300.01	Secondary amine -N-H stretch
	2855.15	Methylene -C-H stretch
	1680.02	Amide -C=O stretch
	1616.40	Aromatic -C=C- stretch
	1090.02	Aromatic -C-Cl stretch
3d	3382.29	Secondary amine -N-H stretch
	3220.27	Phenol -O-H stretch
	3200.98	Hydroxyl-O-H stretch
	3032.20	Aromatic -C-H stretch
	2900.02	Methylene -C-H stretch
	1680.01	Amide -C=O stretch
	1607.72	Aromatic -C=C- stretch
	650.01	Phenol -O-H bend

CODE	ABSORBANCE cm^{-1}	GROUPS
3e	3550.04	Secondary amine -N-H stretch
	3280.11	Phenolic –O-H stretch
	3200.00	Hydroxyl-O-H stretch
	3050.01	Aromatic -C-H stretch
	2900.03	Methylene -C-H stretch
	1680.03	Amide -C=O stretch
	1640.01	Imino -C=N stretch
	1600.02	Aromatic -C=C- stretch
	650.26	Phenolic –O-H bend
3f	3500.03	Hydroxyl -O-H stretch
	3200.01	Secondary amine -N-H stretch
	2918.40	Methylene -C-H stretch
	1690.02	Amide -C=O stretch
	1640.01	Imino -C=N stretch
	1596.15	Aromatic -C=C- stretch
	1525.74	Aromatic nitro -N=O stretch
3g	3116.00	Hydroxyl -O-H stretch
	3200.01	Secondary amine -N-H stretch
	2920.00	Methylene -C-H stretch
	1690.02	Amide -C=O stretch
	1627.01	Imino -C=N stretch
	1607.72	Aromatic -C=C- stretch
	1527.67	Aromatic nitro -N=O stretch
3h	3200.03	Secondary amine -N-H stretch
	3050.01	Aromatic -C-H stretch
	1680.05	Amide -C=O stretch
	1612.54	Aromatic -C=C- stretch
	1230.01	Mono sub. Aromatic -C-F Stretch
	3500.03	Hydroxyl -O-H stretch

CODE	ABSORBANCE cm^{-1}	GROUPS
3i	3300.01	Secondary amine -N-H stretch
	2925.15	Methyl -C-H stretch
	2800.01	Methylene -C-H stretch
	1700.02	Amide -C=O stretch
	1640.02	Imino -C=N stretch
	1602.90	Aromatic -C=C- stretch
	1250.88	Asymmetric -C-O-C- stretch
3j	3449.50	Secondary amine -N-H stretch
	3234.94	Phenol -O-H stretch
	3174.86	Hydroxyl –O-H stretch
	2958.90	Methyl -C-H stretch
	2850.01	Methylene -C-H stretch
	1680.01	Amide -C=O stretch
	1615.44	Aromatic -C=C- stretch
	1256.67	Asymmetric -C-O-C- stretch
	650.02	Phenol -O-H bend
3k	3250.03	Secondary amine -N-H stretch
	3040.01	Aromatic -C-H stretch
	2921.00	Methylene -C-H stretch
	1635.00	Imino -C=N stretch
	1560.04	Thiones -C=S stretch
	1625.12	Aromatic -C=C- stretch
	1089.82	Aromatic -C-Cl stretch
3l	3250.04	Secondary amine -N-H stretch
	3050.04	Aromatic -C-H stretch
	2910.01	Methylene -C-H stretch
	1640.40	Keto -C=O stretch
	1740.00	Imino -C=N- stretch
	1624.01	Aromatic -C=C- stretch
	1572.00	Thiones -C=S stretch
	683.73	Phenol -O-H bend

CODE	ABSORBANCE cm^{-1}	GROUPS
3m	3236.66	Hydroxyl -O-H stretch
	3450.01	Secondary amine -N-H stretch
	1737.92	Keto -C=O stretch
	1559.50	Thiones -C=S stretch
	1050.01	Aromatic -C-Br Stretch

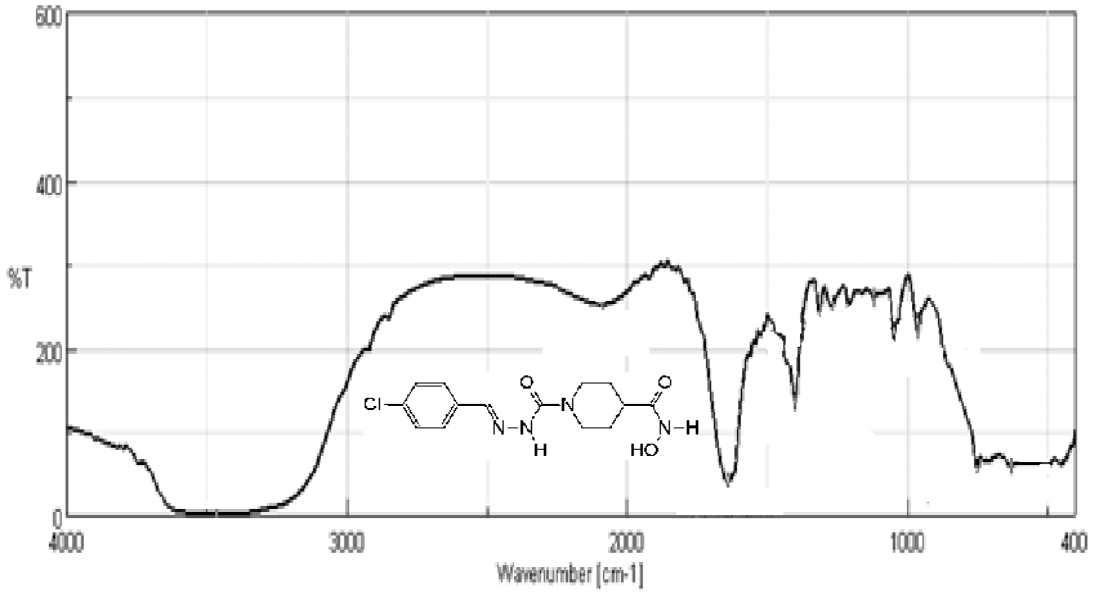


Fig .13 IR spectrum of compound 3a

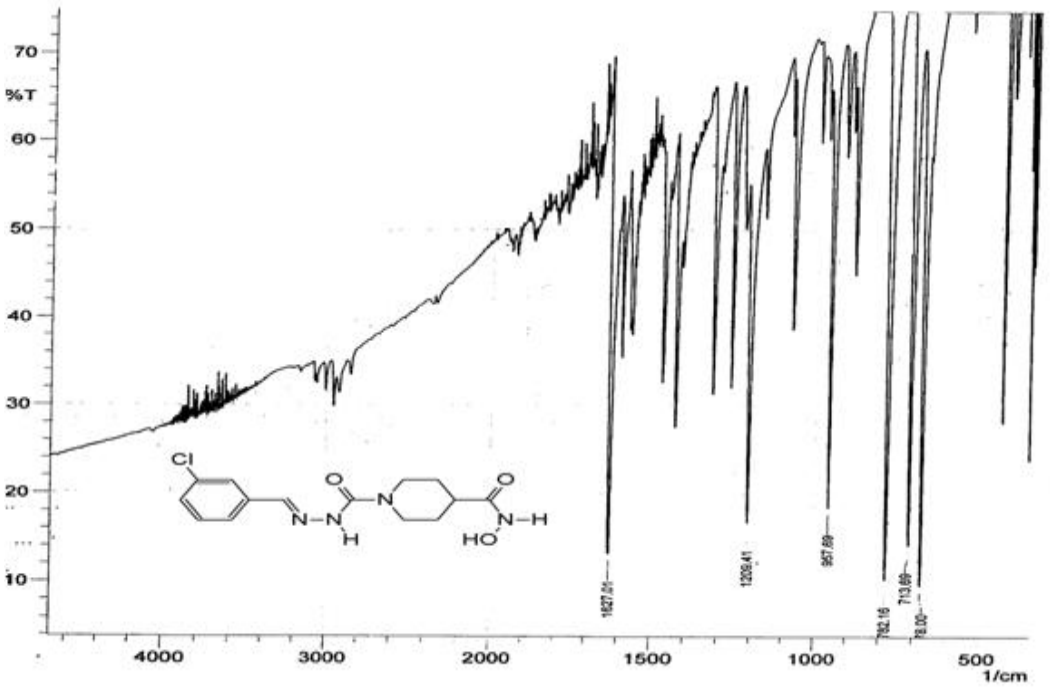


Fig .14 IR spectrum of compound 3b

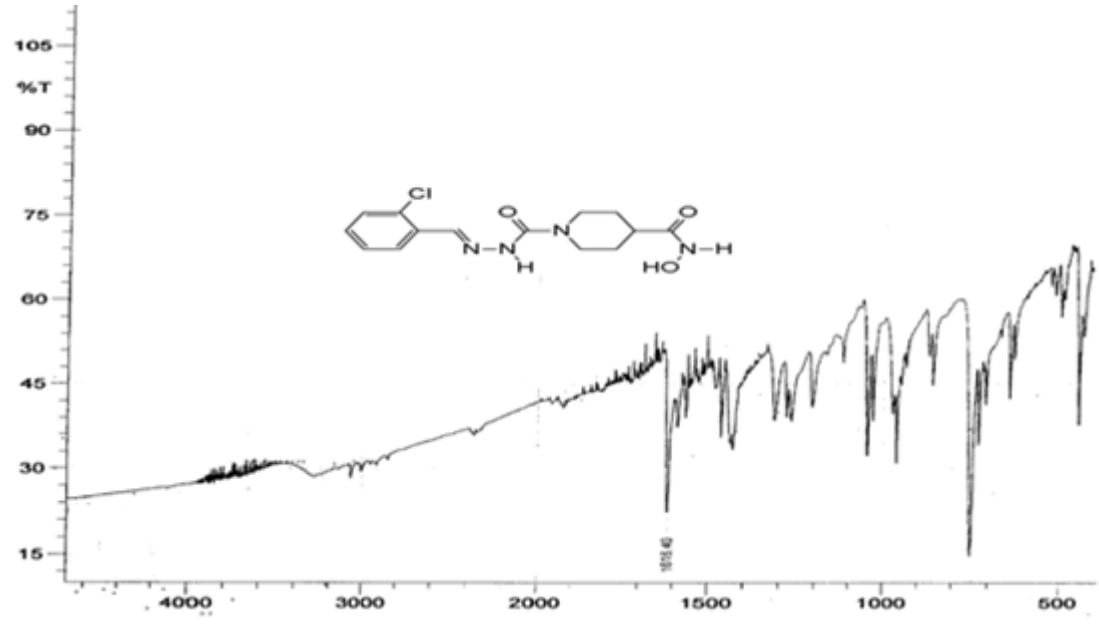


Fig .15 IR spectrum of compound 3c

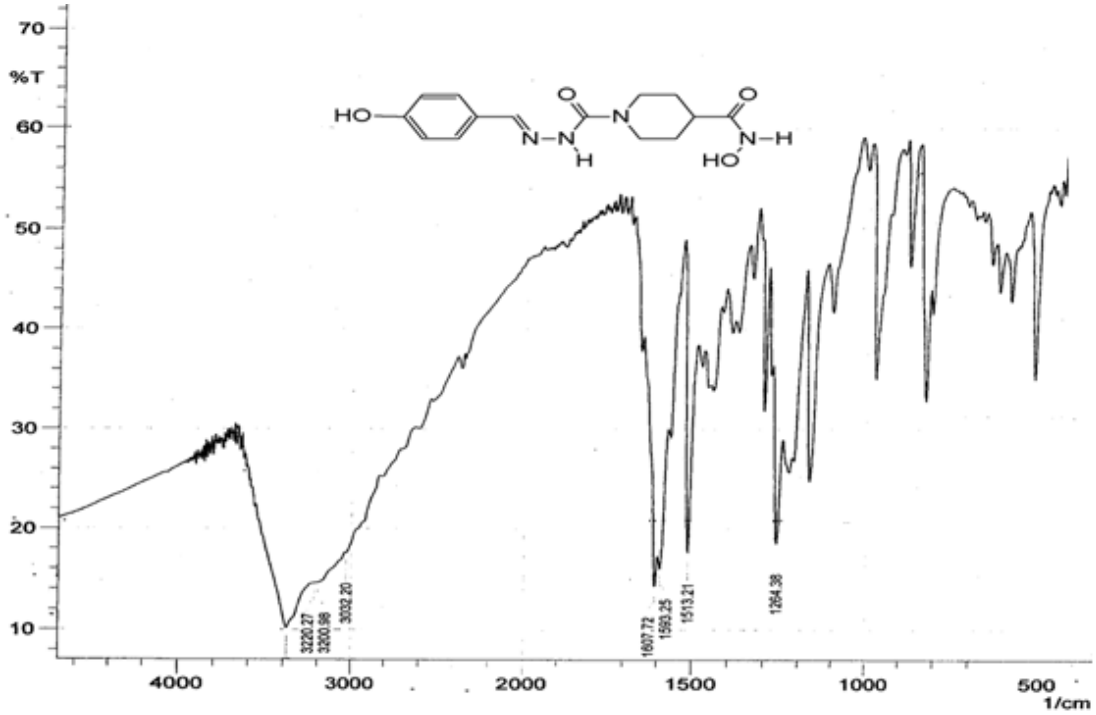


Fig .16 IR spectrum of compound 3d

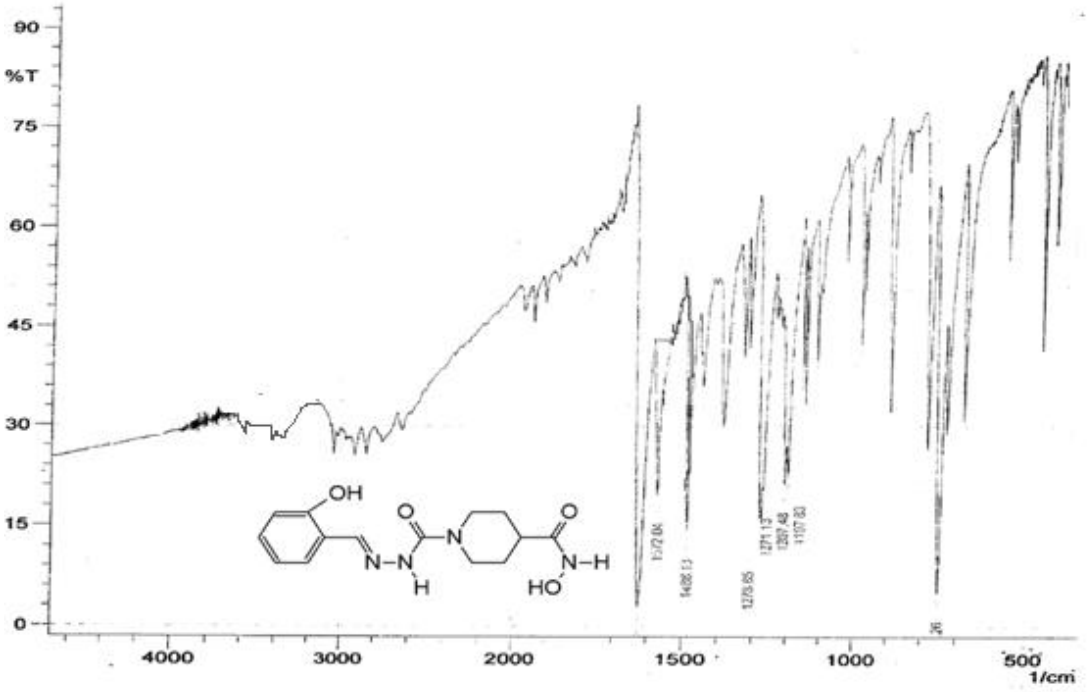


Fig .17 IR spectrum of compound 3e

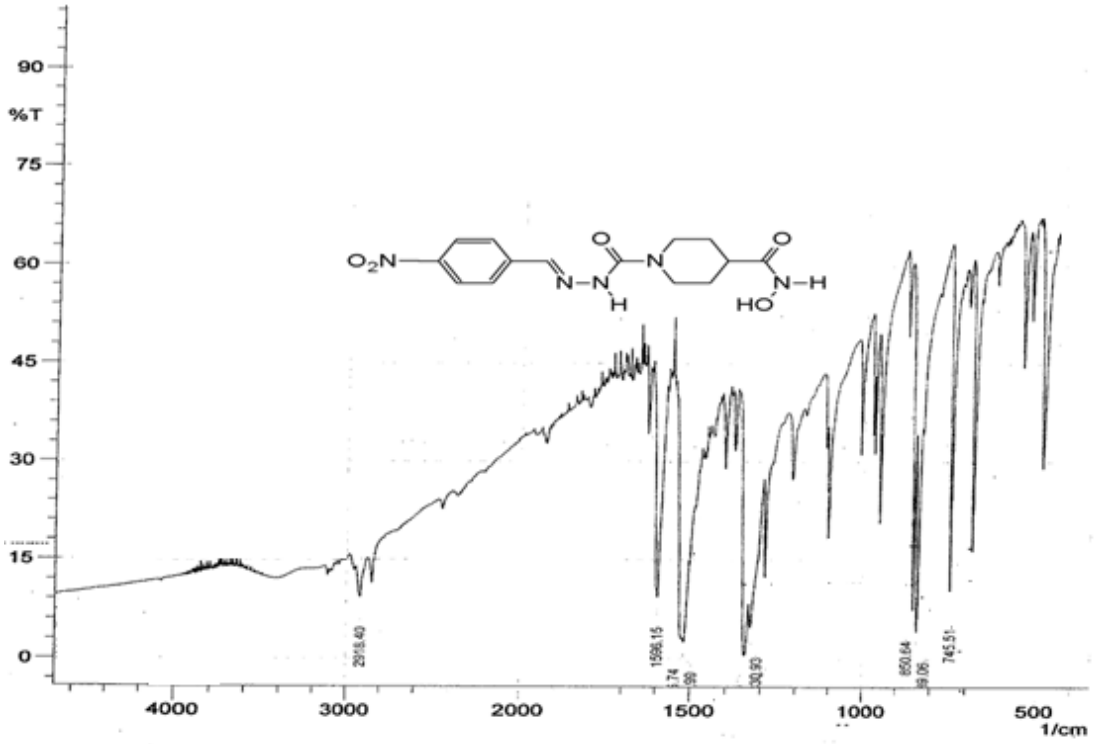


Fig .18 IR spectrum of compound 3f

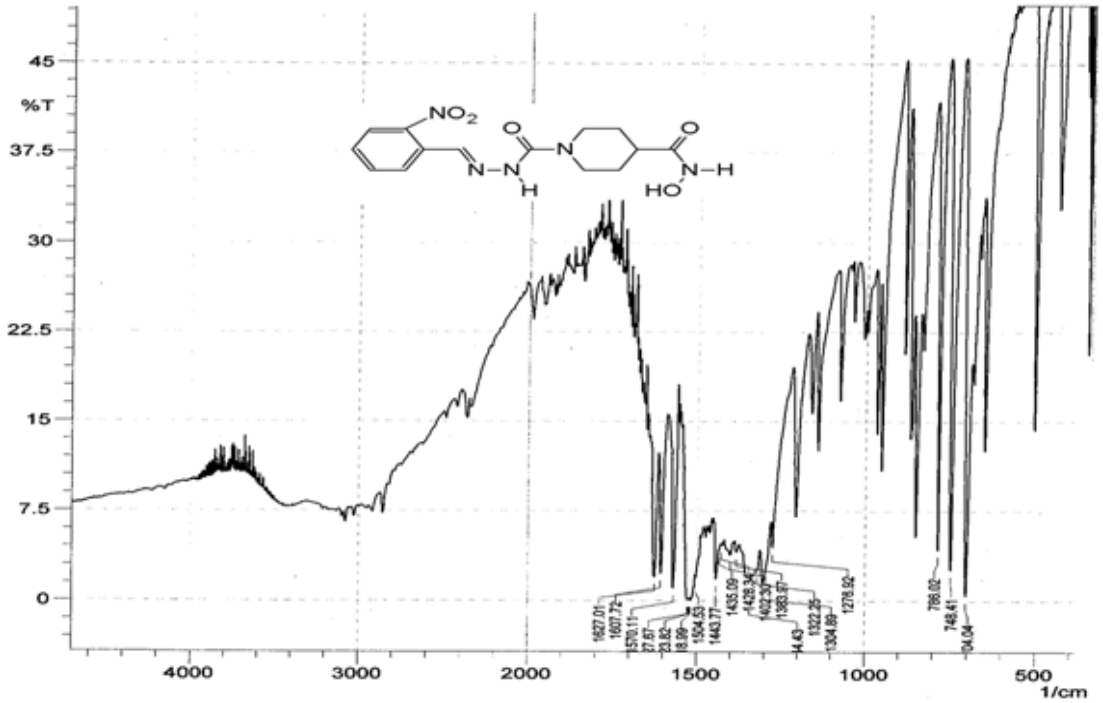


Fig .19 IR spectrum of compound 3g

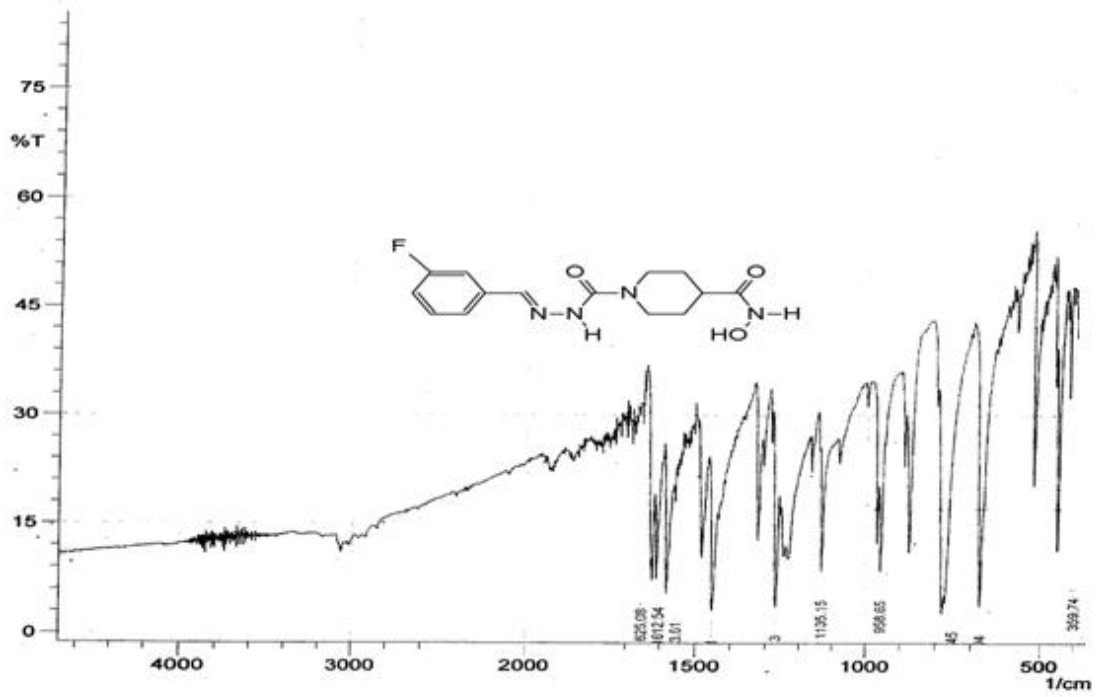


Fig .20 IR spectrum of compound 3h

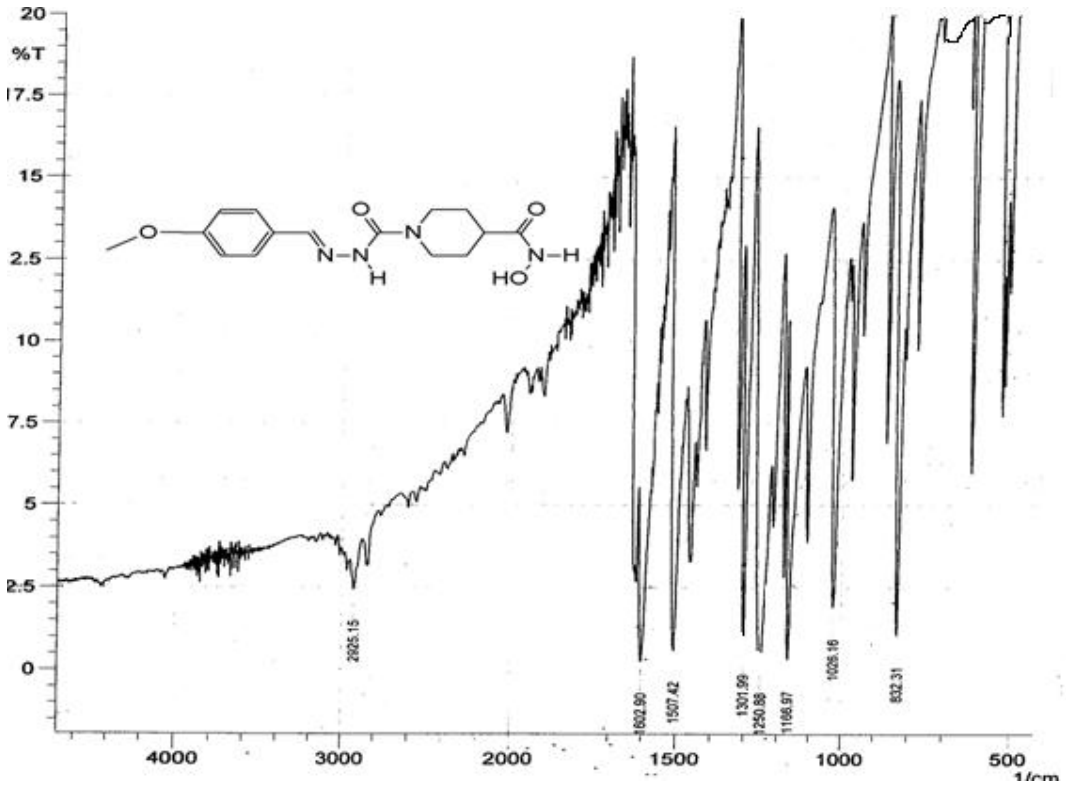


Fig .21 IR spectrum of compound 3i

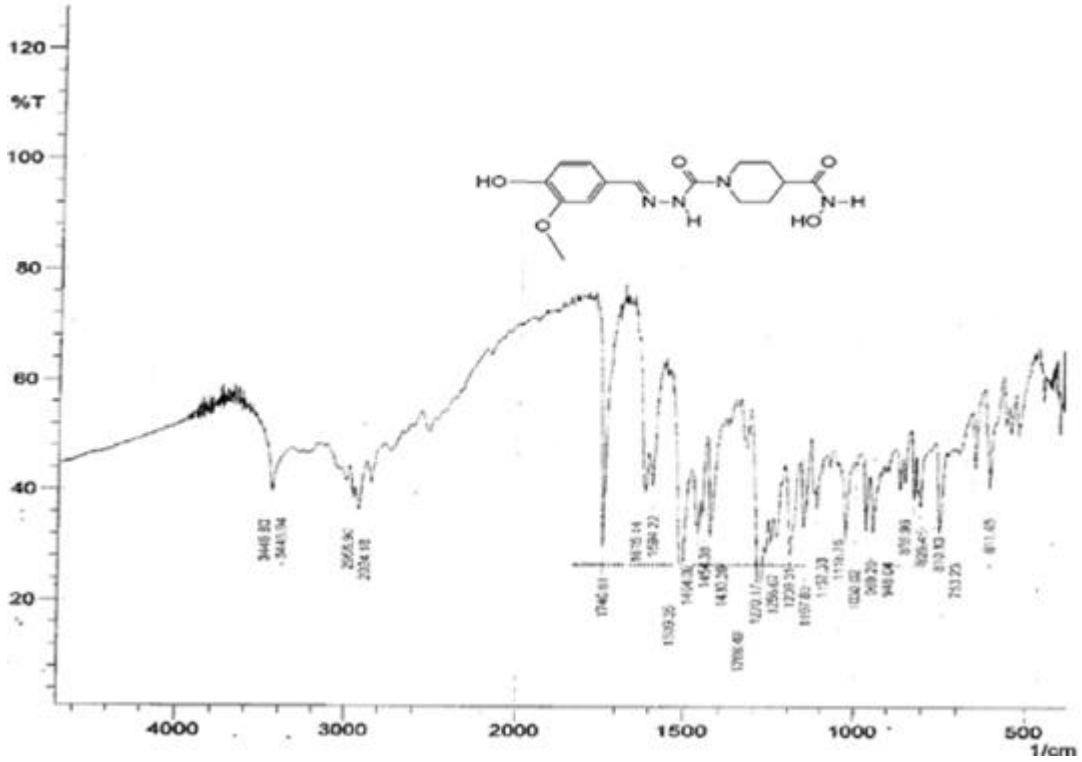


Fig .22 IR spectrum of compound 3j

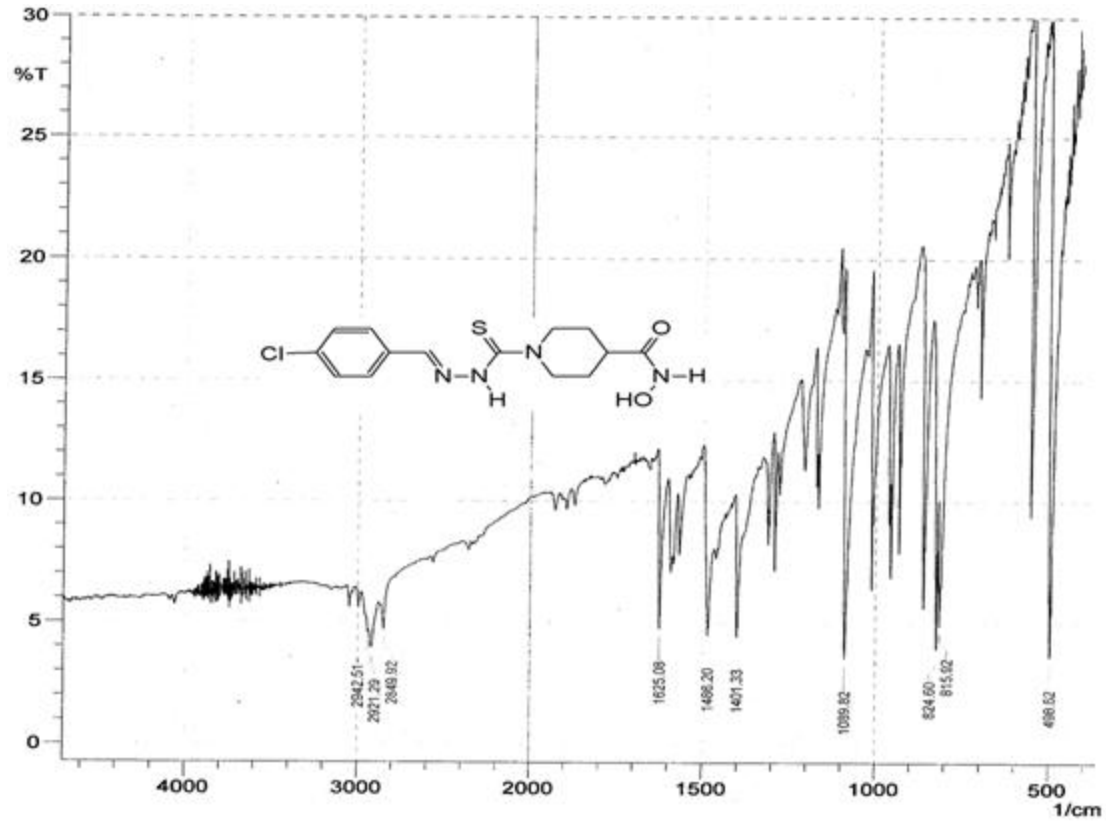


Fig .24 IR spectrum of compound 3k

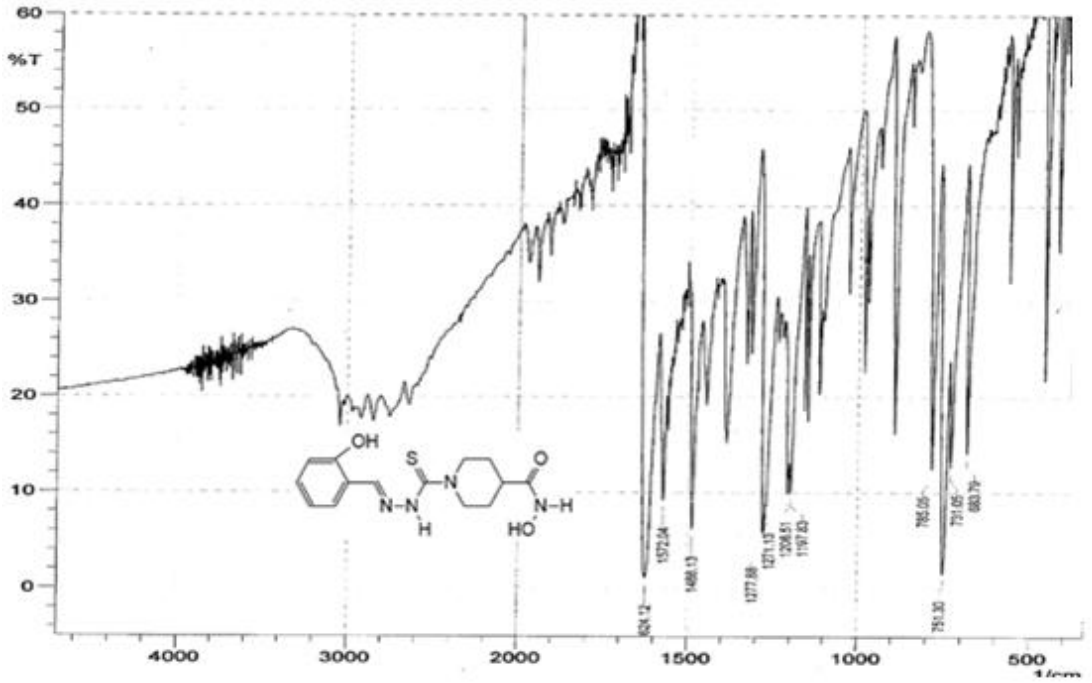


Fig .23 IR spectrum of compound 3l

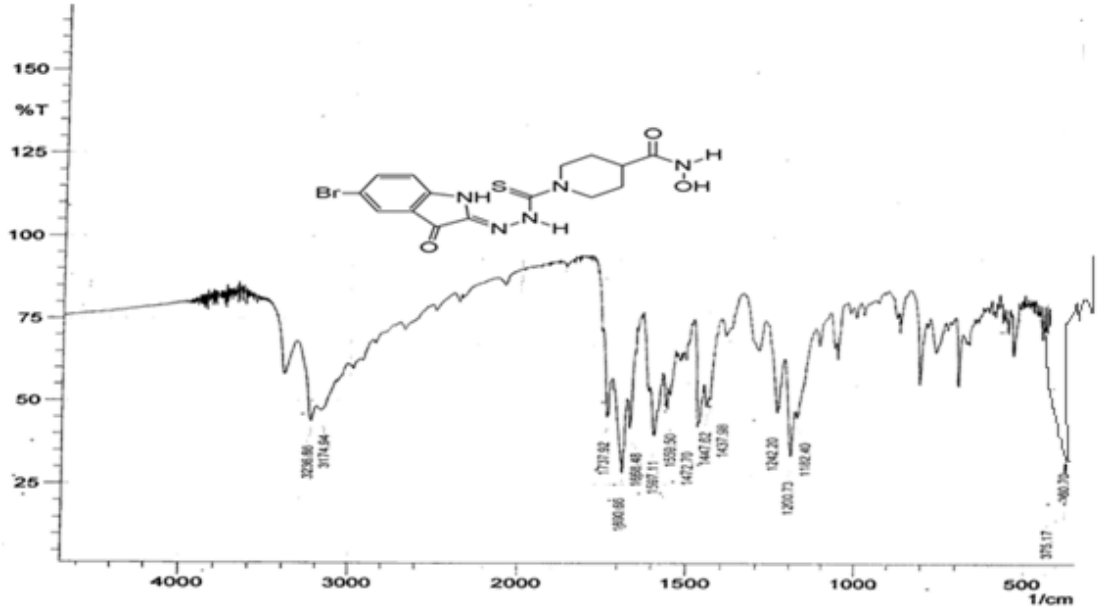


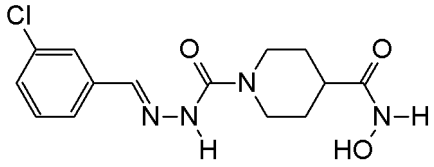
Fig .25 IR spectrum of compound 3m

3.5 NUCLEAR MAGNETIC RESONANCE SPECTROMETRY

Nuclear magnetic spectrometry is an important tool for determining the structures of a molecule. NMR spectrum can give almost unbelievable detailed information about molecular structure:

1. The number of signals: which tell us how many different kinds of protons there are in a molecule?
2. The positions of the signals, which tell us something about the electronic environment of each kind of proton.
3. The intensities of the signals, which tell us how many protons of each kind there are; and the splitting of a signal into several peaks, which tell us about the environment of a proton with respect to other, nearby protons. NMR spectral study was done on Bruker Fourier, Transform-NMR Spectrometer on selected compounds.

3b:1-{[(2*E*)-2-(3-chlorobenzylidene) hydrazinyl] carbonyl}-*N*-hydroxypiperidine-4-carboxamide



8.7 (s, 1H,-NH-OH)

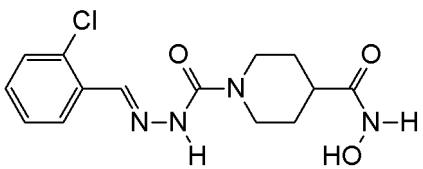
7.3–7.7 (m, 4H, Ar-H),

7.9-8 (s, 1H, CH)

3.2-3.4 (s, 1H, NH)

2.1-2.6 (m, 9H, H in piperidine)

3c: 1-{[(2*E*)-2-(2-chlorobenzylidene) hydrazinyl] carbonyl}-*N*-hydroxypiperidine-4-carboxamide



11.65 (s, 1H,-NHOH)

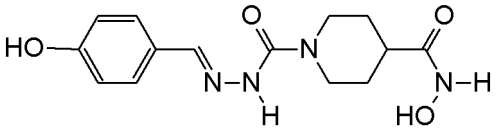
8.9 (s, 1H, Ar-CH=)

8.7-7.3 (m, 4H, ArH)

3.31 (s, IH, CONH)

2.1-2.6 (m, 9H, H in piperidine)

3d: *N*-hydroxy-1-{[(2*E*)-2-(4-hydroxybenzylidene) hydrazine] carbonyl} piperidine-4-carboxamide



10.09 (s, 1H, NHOH)

8.54 (s, 1H, Ar-CH=)

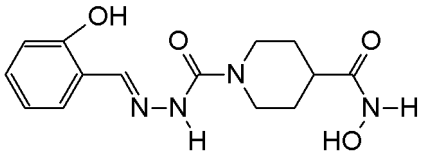
7.69-6.85 (m, 4H, ArH)

3.4 (s, 1H, CONH)

2.5-2.49 (m, 9H, H in piperidine))

2.08 (s, 1H, OH)

3e: *N*-hydroxy-1-{[(2*E*)-2-(2-hydroxybenzylidene) hydrazinyl] carbonyl} piperidine-4-carboxamide



11.11 (s, 1H, Ar OH)

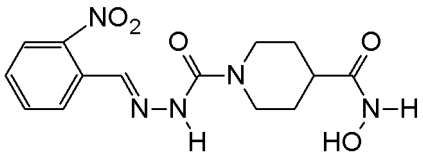
9.04 (s, 1H -NH-OH)

7.7-6.9 (m, 4H, ArH)

3.30 (s, 1H, CONH)

2.5-2.49 (m, 9H, H in piperidine)

3g: *N*-hydroxy-1-{[(2*E*)-2-(2-nitrobenzylidene) hydrazinyl] carbonyl} piperidine-4-carboxamide



8.96 (s, 1H, -NH-OH)

8.3 (s, 1H, Ar-CH=)

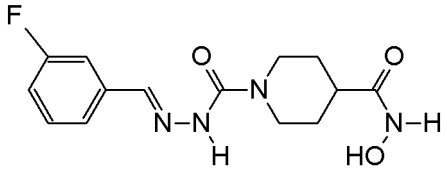
7.9-7.7 (m, 4H, ArH)

3.30 (s, IH, CONH)

2.52-2.49 (m, 9H, H in piperidine)

2.08 (s, 1H, -OH)

3h: 1-{[(2*E*)-2-(3-fluorobenzylidene) hydrazinyl] carbonyl}-*N*-hydroxypiperidine-4-carboxamide



8.72 (s, 1H,-NH-OH)

7.74-7.36 (m, 4H, ArH)

3.33 (s, 1H,-CONH)

2.5-2.49 (m, m, 9H, H in piperidine)

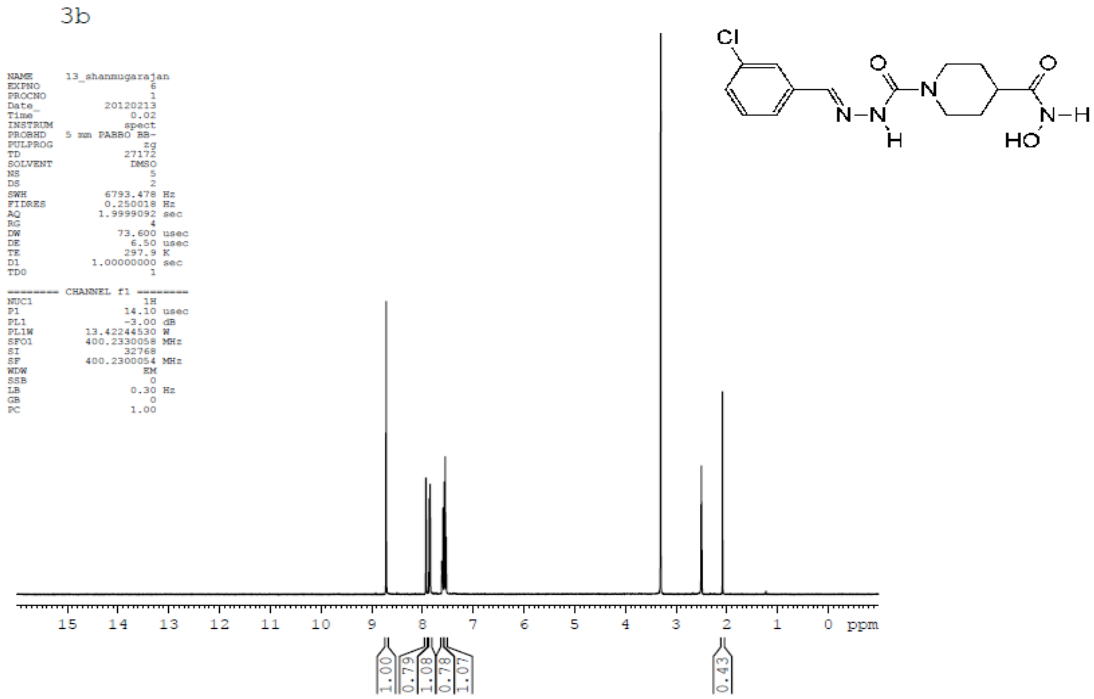


Fig .26 ^{1}H NMR spectrum of compound 3b

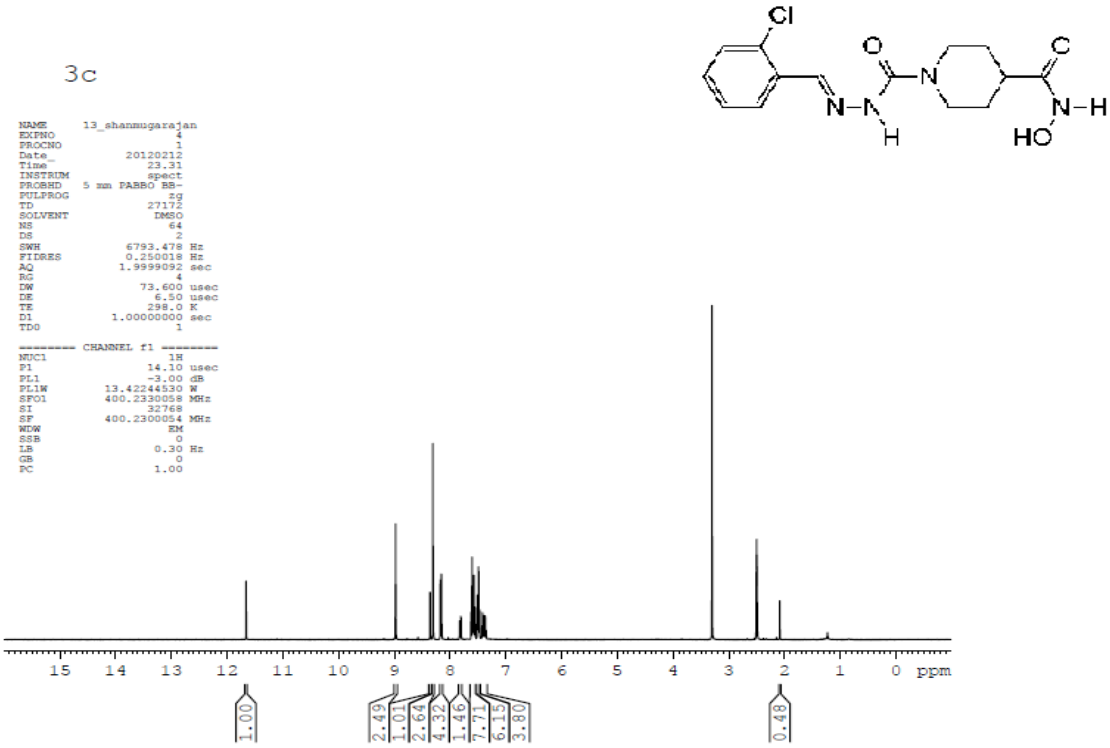


Fig.27 ^{1}H NMR spectrum of compound 3c

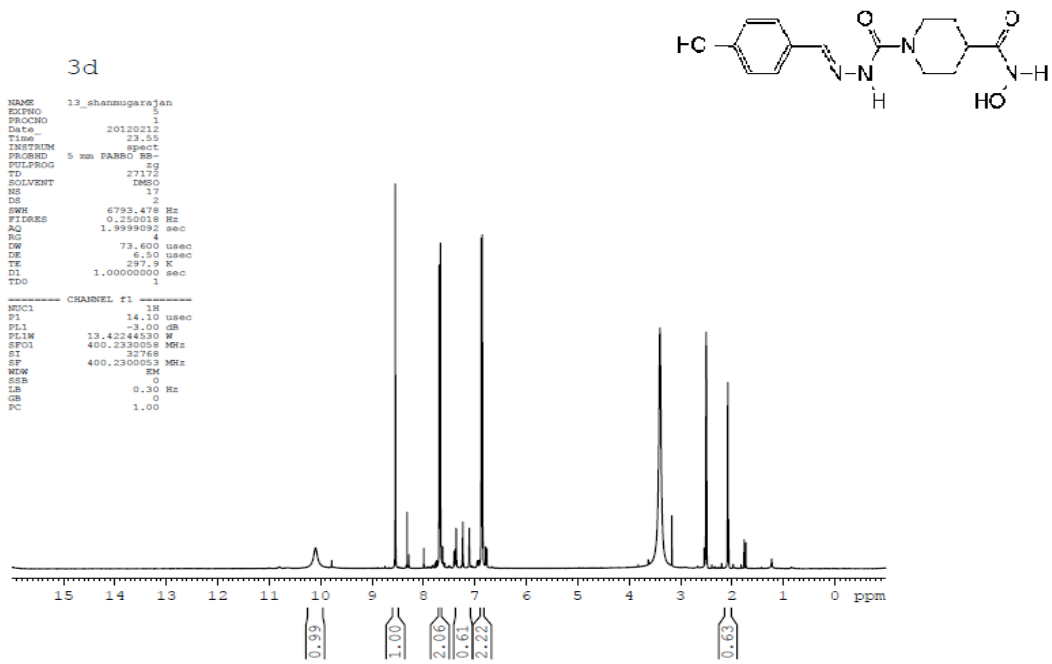


Fig .28 ^{1}H NMR spectrum of compound 3d

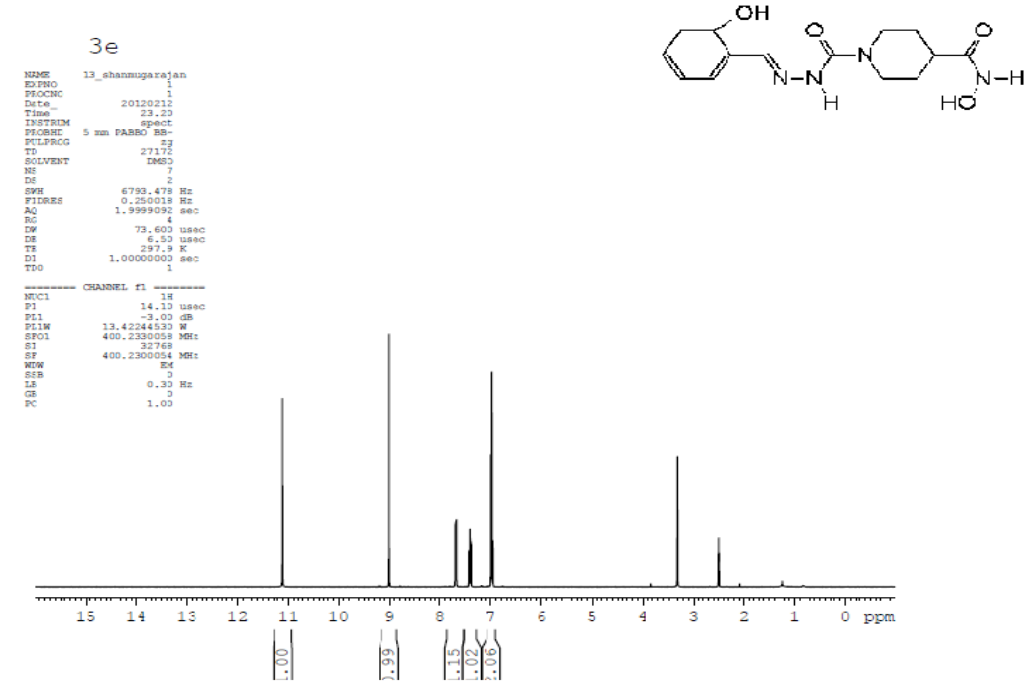


Fig .29 ^{1}H NMR spectrum of compound 3e

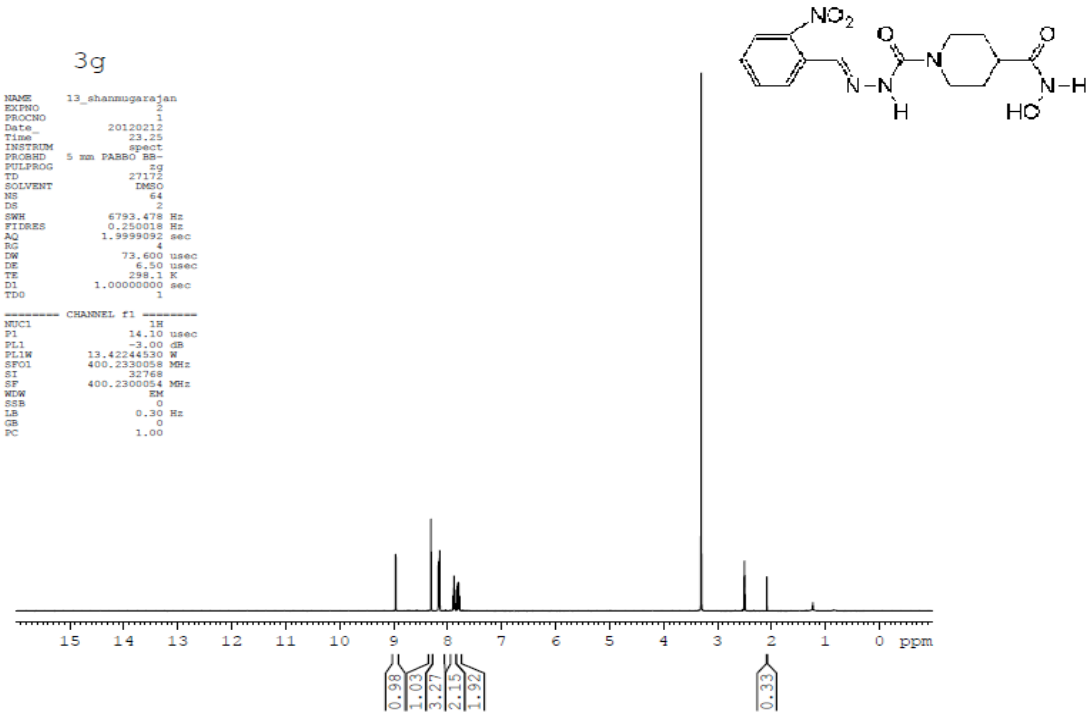


Fig. 30 ^{1}H NMR spectrum of compound 3g

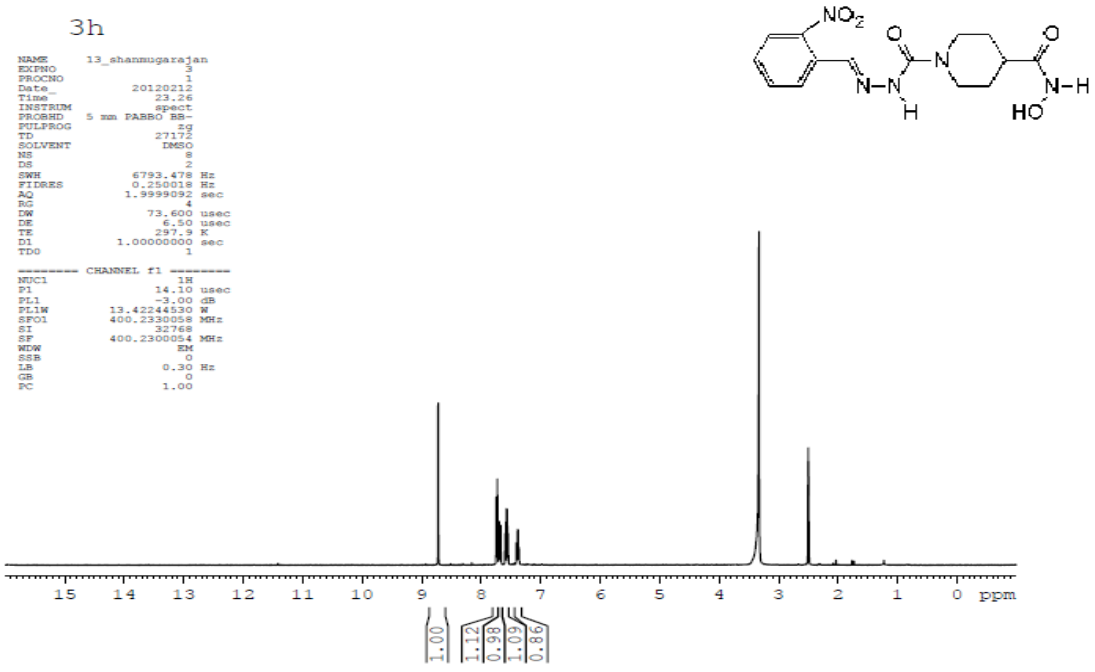


Fig .31^{1}H NMR spectrum of compound 3h

3.5. MASS SPECTROSCOPY

In the technique of mass spectroscopy, the compound under investigation is bombarded with a beam of electrons which produce an ionic molecule or ionic fragments of the original species. The resulting assortment of charged particles is then separated according to their masses. The spectrum produced, known as mass spectrum is a record of information regarding various masses produced and their relative abundance.

The mass spectra of selectively newly synthesized compounds were recorded on JEOL GCMATE instrument.

Table. 16

S.N.	Compound code	Molecular mass(m\z)	Base peak	Molecular ion peak
1	3e	306.32	251.33	306.49
2	3g	335.32	251.33	335.93
3	3l	332.38	146.51	332.38

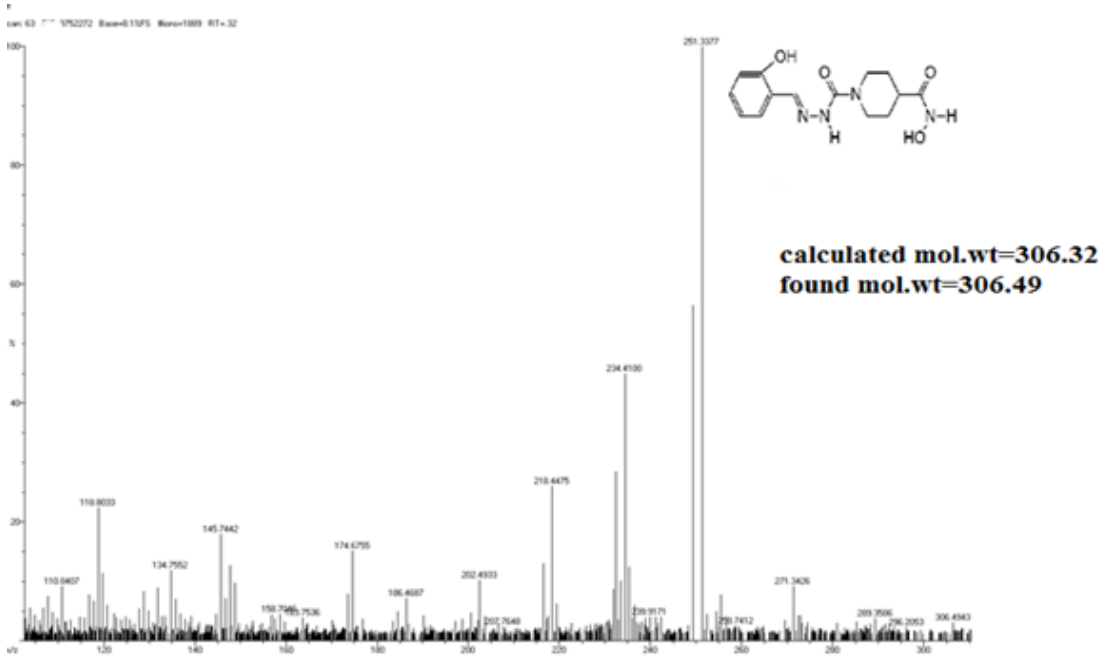


Fig .32 Mass spectrum of compound 3e

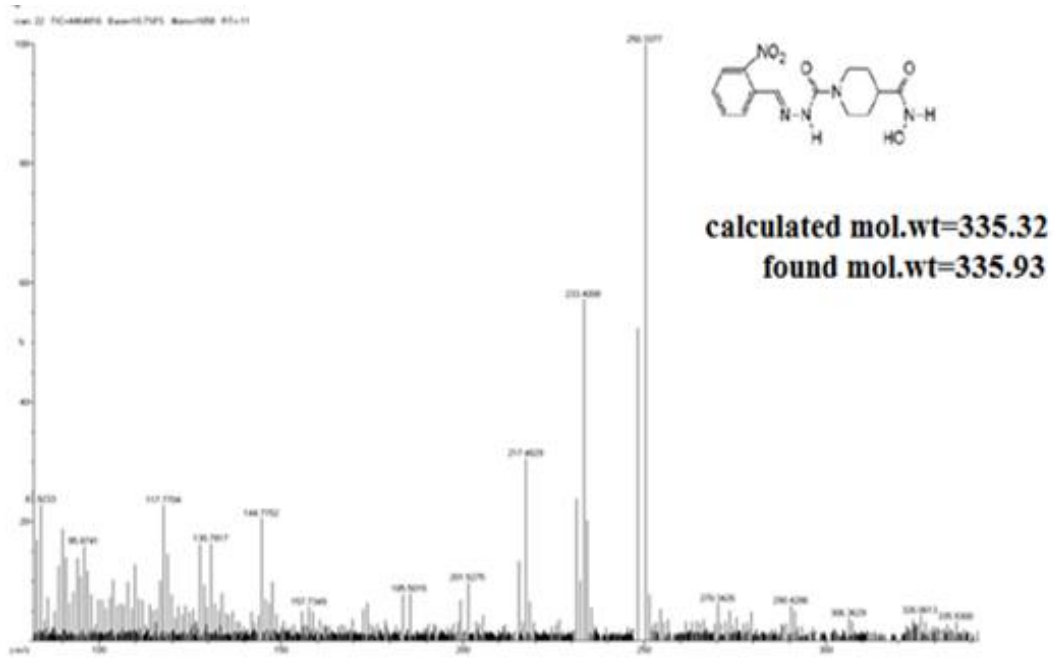


Fig .33 Mass spectrum of compound 3g

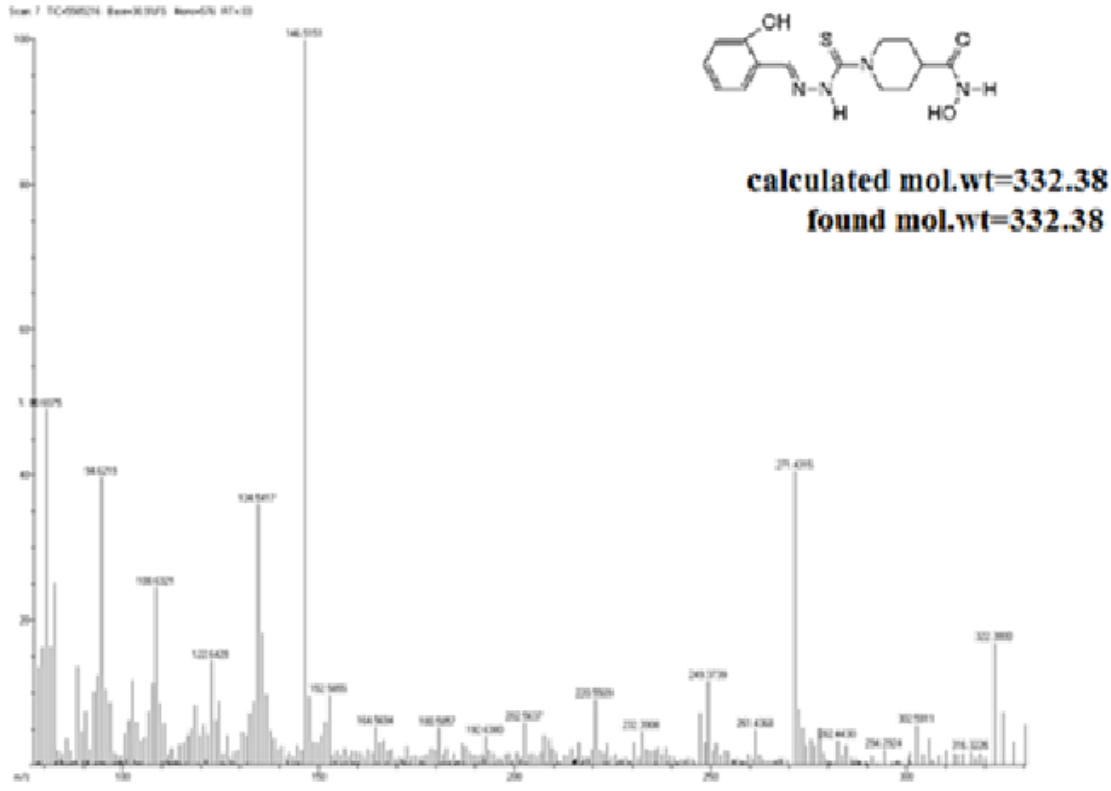


Fig .34 Mass spectrum of compound 3l

IN VITRO ANTI CANCER ACTIVITY [4&7]

The human cervical cancer cell line (HeLa) was obtained from National Centre for Cell Science (NCCS), Pune, and grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS).

For screening experiment, the cells were seeded into 96-well plates at plating density of 10, 000 cells/well and incubated to allow for cell attachment at 37^0C, 5% CO_2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the samples. The samples were initially dissolved in dimethylsulphoxide (DMSO) and further diluted in serum free medium to produce five concentrations. One hundred microlitres per well of each concentration was added to plates to obtain final concentrations of 100, 50, 25, 12.5 and 6.25 µM. The final volume in each well was 200 µl and the plates were incubated at 37^0C, 5% CO_2, 95% air and 100% relative humidity for 48h. The medium containing without samples were served as control. Triplicate was maintained for all concentrations.

After 48h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^0C for 4h. The medium with MTT was then flicked off and the formed Formosan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula.

% cell Inhibition = 100- Abs (sample)/Abs (control) x100.

Nonlinear regression graph was plotted between % Cell inhibition and Log_{10} concentration and IC_{50} was determined using Graph Pad Prism software

- All synthesized compounds were screened against screened (HeLa)human cervical cancer cell line at different concentration

***In vitro* cytotoxicity against (HeLa) human cervical cancer cell line**

Table.17

Code	Cell line	Concentration (µM)	% cell inhibition
3a	HeLa	0.1	3.769401
		1	6.430155
		10	15.74279
		100	24.68588
3c	HeLa	0.1	0.723327
		1	7.323689
		10	17.26944
		100	58.13743
3d	HeLa	0.1	5.786618
		1	12.65823
		10	17.35986
		100	36.52803
3e	HeLa	0.1	2.512934
		1	10.42129
		10	28.82483
		100	33.1116
3f	HeLa	0.1	5.838877
		1	8.57354
		10	9.164819
		100	22.32077
3h	HeLa	0.1	-1.33038
		1	3.473762
		10	7.169254
		100	18.62528
3i	HeLa	0.1	1.847746
		1	5.912786
		10	8.056171
		100	24.68588

Code	Cell line	Concentration (µM)	% cell Inhibition
3j	HeLa	0.1	3.769401
		1	6.430155
		10	15.74279
		100	24.68588
3k	HeLa	0.1	3.204272
		1	10.48064
		10	32.84379
		100	88.251
3l	HeLa	0.01	3.627968
		0.1	12.26913
		1	32.38786
		10	54.8153
3m	HeLa	0.1	6.675567
		1	30.70761
		10	71.49533
		100	99.3992

***In vitro* cytotoxicity (IC_{50} (µM)) against (HeLa) human cervical cancer cell line**

S.NO	CODE	IC_{50} (µM)
1	3c	66.95 µM
2	3k	17.84 µM
3	3l	5.83 nM
4	3m	2.836 µM

Table .18

***Invitro* anticancer activity of compound 3c on HeLa cell line**

Conc	0.1 µM	1 µM	10 µM	100 µM	Cont
ABS	0.374	0.332	0.306	0.143	0.388
	0.36	0.335	0.304	0.166	0.359
	0.364	0.358	0.305	0.154	0.359
Avg	**0.366**	**0.341667**	**0.305**	**0.154333**	**0.368667**

Table .19

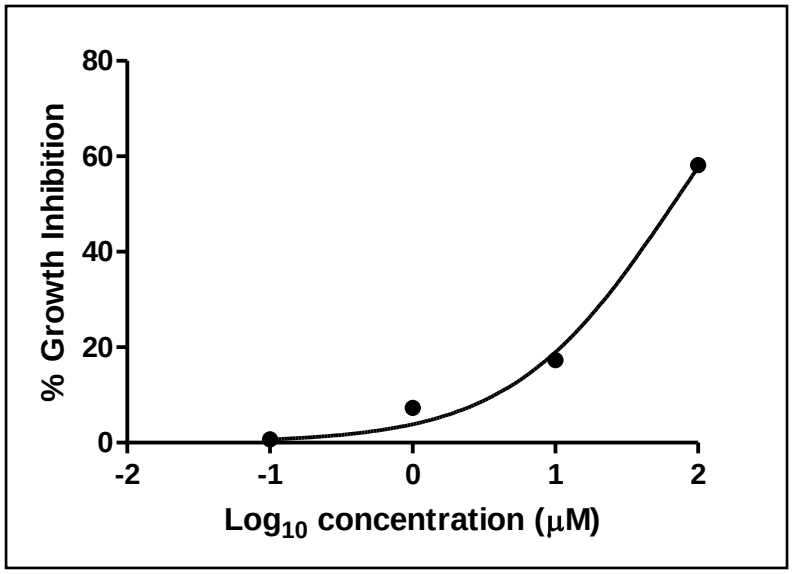
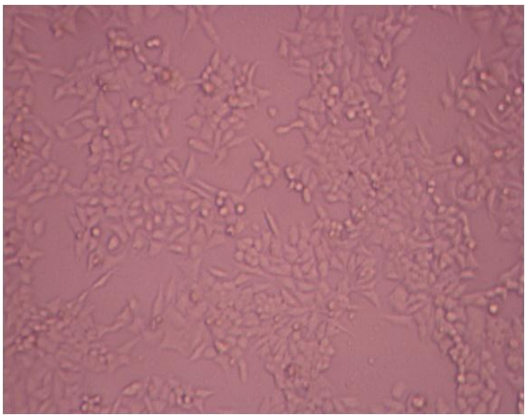
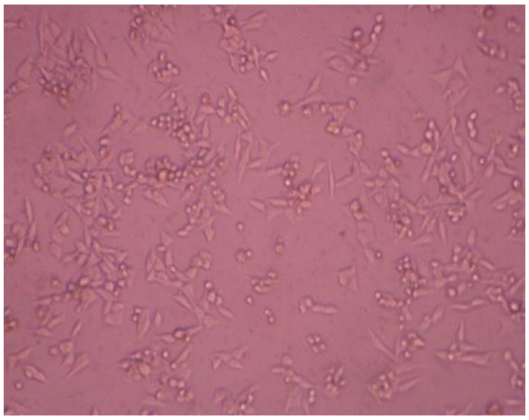


Fig .35 % growth inhibition of compound 3c

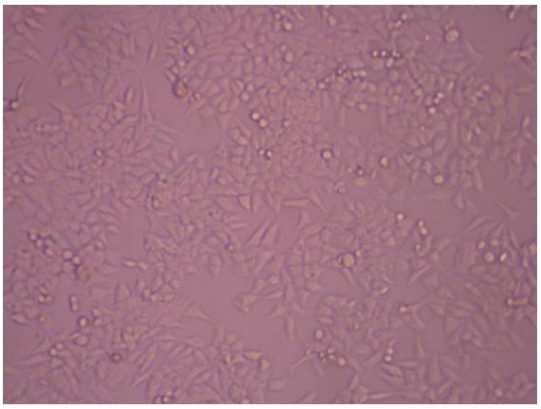
***Invitro* anticancer activity of compound 3c on HeLa cell line**



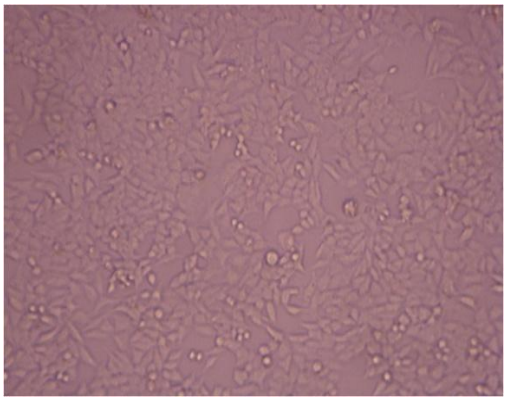
0.1µM



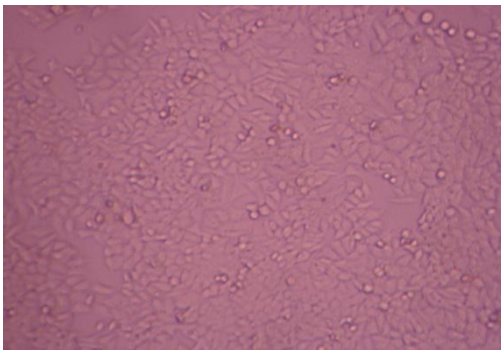
1µM



10 µM



100 µM



control

Fig.39 HeLa cell line inhibition by the compound 3c

***Invitro* anticancer activity of compound 3k on HeLa cell line**

Conc	0.1 µM	1 µM	10 µM	100 µM	Cont
ABS	0.481	0.329	0.121	0.002	0.492
	0.461	0.34	0.154	0.005	0.488
	0.456	0.369	0.152	0.002	0.518
Avg	**0.466**	**0.346**	**0.142333**	**0.003**	**0.499333**

Table.20

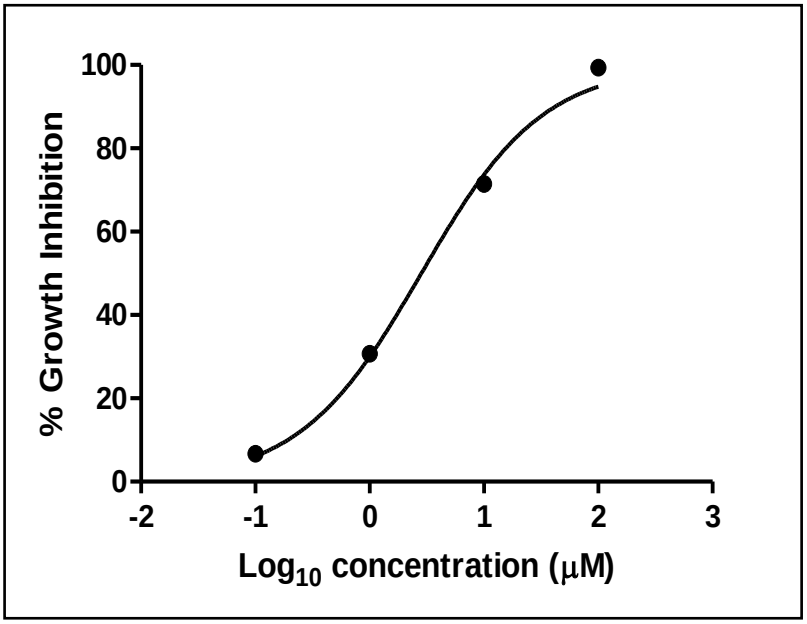
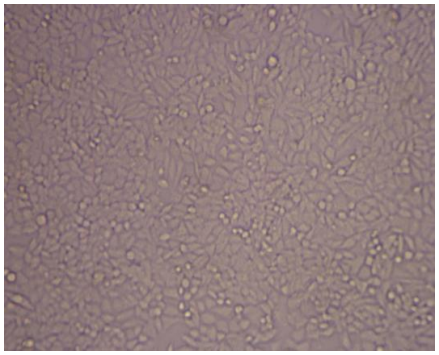
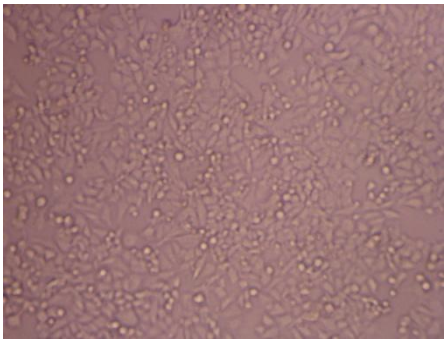


Fig .36 % growth inhibition of compound 3k

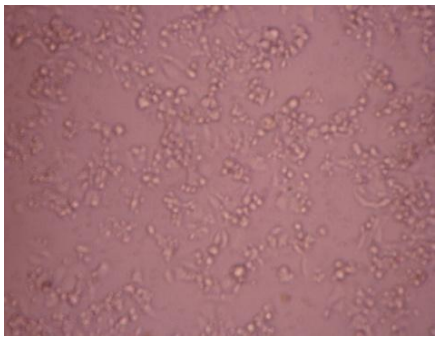
***Invitro* anticancer activity of compound 3k on HeLa cell line**



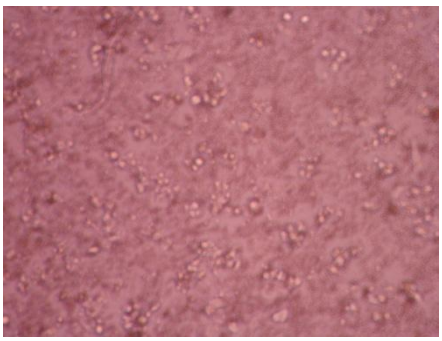
0.1µM



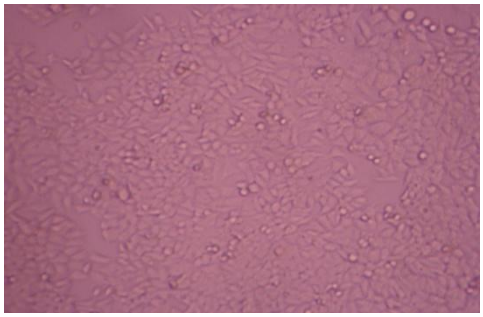
1µM



10 µM



100 µM



Control

Fig. 40 HeLa cell line inhibition by the compound 3k

***Invitro* anticancer activity of compound 3l on HeLa cell line**

Conc	0.1 µM	1 µM	10 µM	100 µM	Cont
ABS	0.494	0.444	0.34	0.054	0.492
	0.486	0.445	0.347	0.069	0.488
	0.47	0.452	0.319	0.053	0.518
Avg	**0.483333**	**0.447**	**0.335333**	**0.058667**	**0.499333**

Table.21

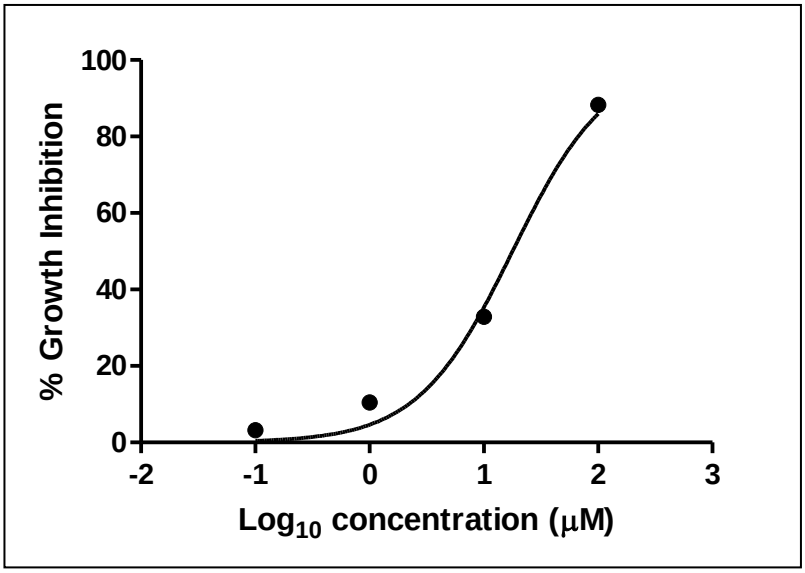
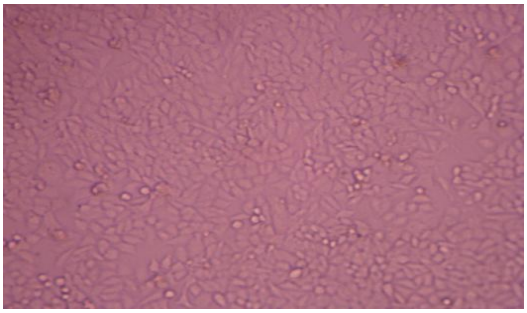
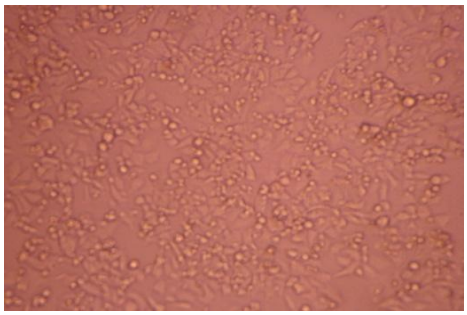


Fig .37 % growth inhibition of compound 3l

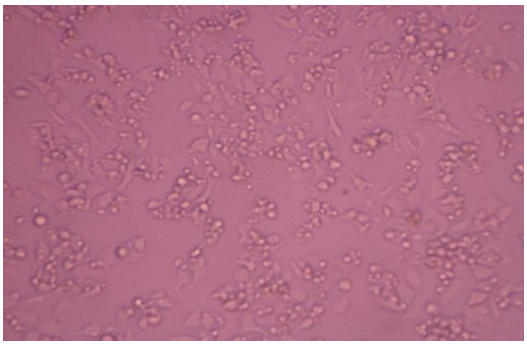
***Invitro* anticancer activity of compound 3l on HeLa cell line**



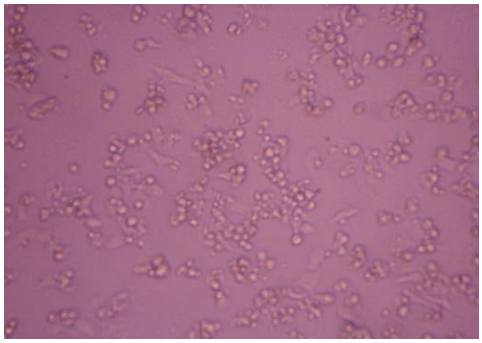
0.1nM



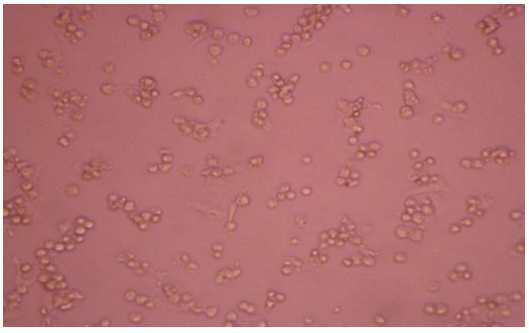
1.0nM



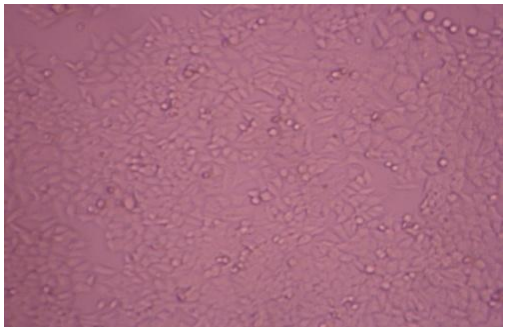
10nM



100nM



1000nM



control

Fig .41 HeLa cell line inhibition by the compound 3l

***Invitro* anticancer activity of compound 3m on HeLa cell line**

Conc	0.1 µM	1 µM	10 µM	100 µM	Cont
ABS	0.481	0.329	0.121	0.002	0.492
	0.461	0.34	0.154	0.005	0.488
	0.456	0.369	0.152	0.002	0.518
Avg	**0.466**	**0.346**	**0.142333**	**0.003**	**0.499333**

Table.22

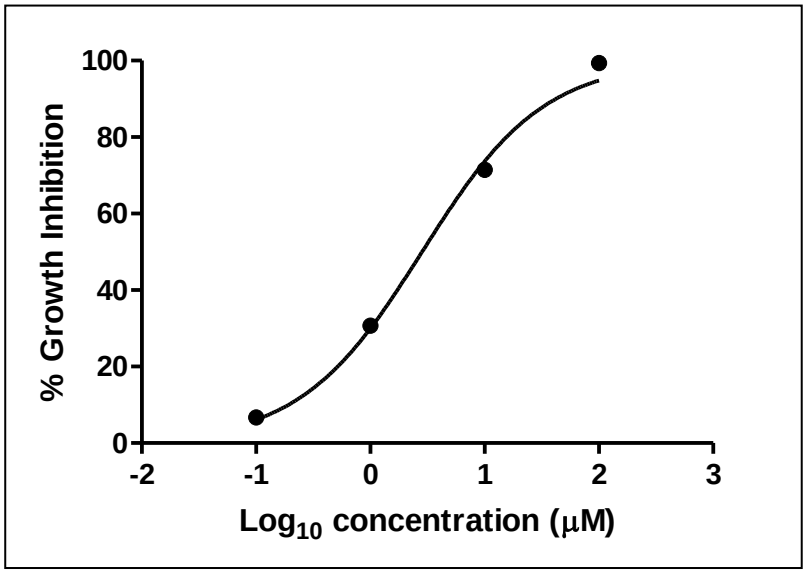
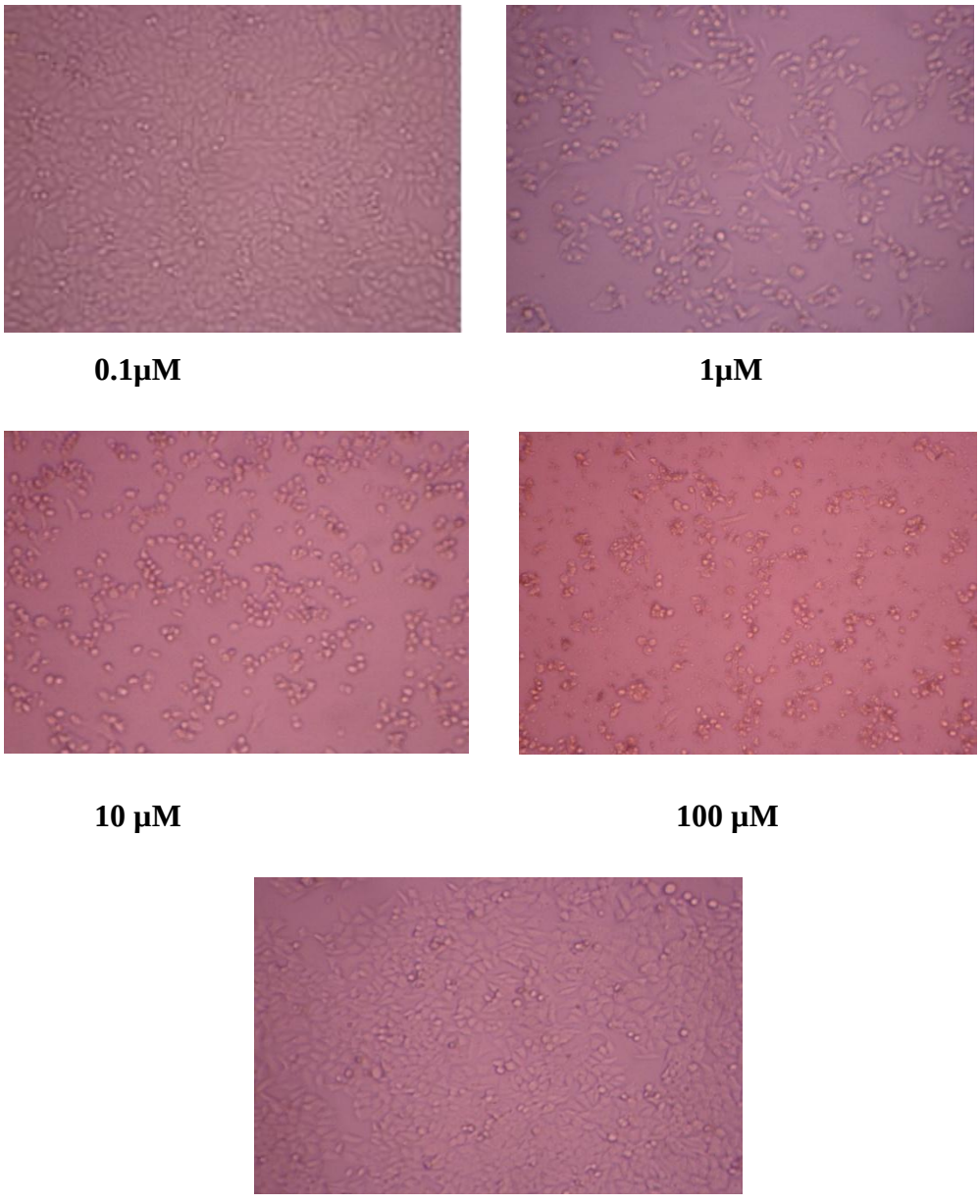


Fig .38 % growth inhibition of compound 3m

***Invitro* anticancer activity of compound 3m on HeLa cell line**



0.1µM **1µM**

10 µM **100 µM**

Control

Fig .42 HeLa cell line inhibition by the compound 3m

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4. RESULTS AND DISCUSSION

Chemistry and Synthesis

Ethyl hydrazinecarboxylate was prepared from ethyl chloroformate based on previously reported procedures. Ethy chloroformate coupled with hydrazine hydrate in presence of triethyl amine to yield Ethyl hydrazinecarboxylate**.** The synthesis of Schiff base of ethyl 2- substituted methylidene hydrazine carboxylate (1a-j) are described in step 2. Compound (I) have been coupled immediately with respective aldehydes in presence of glacial acetic acid to yield substituted ethyl 2- substituted methylidene hydrazine carboxylate. Then it was substitutedd with methyl piperidine -4-corboxylate to yield of substituted methyl 1-(ethylidine aminocarbamoyl) piperidine -4-carboxylate(2a-j) which undergoes base hydrolysis and followed by substituted with hydroxalamine hydrochloride in presence of K_2CO_3 to yield substituted 4-(hydroxycarbamoyl)-N'-ethylidenepiperidine-1-carbohydrazide.

Ethyl carbonochloridodithioate was prepared from ethyl chloroformate which undergoes thionation by using lawessons reagent to yield ethyl carbonochloridodithioate and it was coupled with hydrazine hydrate in presence of triethylamine to yield ethyl hydrazinecarbodithioate. The synthesis of Schiff base of substituted ethyl 2- substituted methylidene hydrazine carbothioate (1k-m) is discribed in step 2. Ethyl hydrazinecarbodithioate have been coupled immediately with respective aldehydes & ketone in presence of glacial acetic acid to yield substituted Schiff's base of ethyl 2-substituted methylidene hydrazine carbothioate. Then it was substitutedd with methyl piperidine -4-corboxylate to yield of substituted methyl (1-methyleneamino thiocarbomyl) piperidine -4-corboxylate (2k-m) which undergoes base hydrolysis and followed by substituted with hydroxalamine hydrochloride in presence of K_2CO_3 to yield substituted Substituted *N*-hydroxy-1-[(2- methylidene hydrazinyl) carbonothioyl] piperidine-4-carboxamide carbohydrazide(3k-m)

Physical properties

Melting point

Melting points were carried in open capillaries on Thomas However melting point apparatus which are uncorrected. A synthesized compounds melting point and its reactant melting points were recorded. A reactants and products melting point were differing from each others. It is clearly indicates that the formation of a new chemical entities. Melting points were given in table-1.

Solubility

Solubility properties of synthesized compounds were determined in various solvents and it was found that all compounds were completely soluble in DMSO, partially soluble in, chloroform and insoluble in water.

Percentage of yield

Percentage of yield of all synthesized compounds was calculated and values are given in the table-1.

C Log P

C Log P was calculated for all newly synthesized compounds and values are given in the table-1.

Thin layer chromatography

Thin layer chromatography was performed for all newly synthesized compound as well as the parent compounds, all synthesized compounds gave a single spot whose R_f values are different from their reactants. It ultimately shows that completion of reaction and purity. The R_f values are given in table-2.

IR-Spectroscopy

Infra red spectroscopy was taken for all newly synthesized compounds. Amide -C=O stretch is absorbed in the region of 1680 cm^{-1} is clearly indicate the step 1.

Methylene -C-H stretch is strongly absorbed in 2900-2936 cm^{-1}, it is clearly gives a chemical entities in step 2. Imino -C=N- stretch was absorbed in the region of 1635-1640 cm^{-1} its shows that formation of Schiff base. Secondary amine –N-H sretch was observed in the region of 3200-3500cm^{-1} it clearly indicating the proceeding of step 4. Thiones -C=S stretch appeared in the region of 1559.50-1560 and other relevant peaks are also appeared, it further indicate the new chemical entities.

^{1}H NMR Spectroscopy

The ^{1}H NMR spectra were taken for selected synthesized compounds (3b, 3c, 3d, 3e, 3g & 3h). 8.2-11.6 δ ppm was appeared in all synthesized compounds. 7.3-7.9 δ ppm multiplet of 4H, Ar-H of benzene ring was appeared in all synthesized compounds. 2.1-2.6 δ ppm multiplet of 9H, Piperidine acyclic proton was appeared in the entire spectrum. 8.3 (s, 1H, -CH) Methyline proton was appeared in the spectrum. 3.30 (s, IH, CONH) amide proton was present in all synthesized compounds. It further estabilished the the structre of compounds.

MASS Spectroscopy

The mass spectral analysis of the synthesized compound 3e, 3g & 3k was performed, and their mass spectrum of the compound was in agreement with its molecular weight, molecular ion and base peaks are given in table-4.

Biological activity

***In vitro* anti tumor evaluation**

The antitumor activity of all compounds against human cervical cancer cell line (HeLa) was determined by MTT assay method. Cell line was incubated with different concentration for each compound and was used to create compound concentration various percentage of cell inhibition. These response parameter (IC_{50}) was calculated for each cell line. The IC_{50} value corresponds to the compound's concentration causing a net 50% loss of initial cells at cell of the incubation period. Compound 3l (*N*-hydroxy-1-{[(2*E*)-2-(2-hydroxybenzylidene) hydrazinyl] carbonothioyl} piperidine-4-carboxamide) was found to be more potent and it shows the IC50 value at 5.83nM.

5. CONCLUSION

A new series of piperidine hydroxamate derivatives have been synthesized and evaluated for their potency as histone deacetylase inhibitors against human cervical cancer cell line (HeLa). Of those, compound 3l (*N*-hydroxy-1-{[(2*E*)-2-(2-hydroxybenzylidene) hydrazinyl] carbonothioyl} piperidine-4-carboxamide) depicted as a potent compound with promising activity and shown IC_{50} value was found to be 5.83nM.

Further the lead compound would be evaluated against a panel of cancer cell lines and establish its possible mechanism through enzyme inhibition assay.